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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification <sup>6</sup> : <b>C12N 15/53, 9/04, 1/21</b>		<b>A1</b>	(11) International Publication Number: <b>WO 98/51802</b> (43) International Publication Date: 19 November 1998 (19.11.98)
(21) International Application Number: <b>PCT/US98/09627</b> (22) International Filing Date: <b>12 May 1998 (12.05.98)</b> (30) Priority Data: <b>60/046,182</b> <b>12 May 1997 (12.05.97)</b> <b>US</b> (63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Application <b>US</b> <b>60/046,182 (CIP)</b> <b>Filed on</b> <b>12 May 1997 (12.05.97)</b> (71) Applicant (for all designated States except US): <b>THERMOGEN, INC. [US/US]; 2201 W. Campbell Park Drive, Chicago, IL 60612 (US).</b> (72) Inventors; and (75) Inventors/Applicants (for US only): <b>DEMIRJIAN, David, C. [US/US]; Chicago, IL 60615 (US). BRIKUN, Igor, A. [US/US]; Forest Park, IL 60130 (US). CASADABAN, Malcolm, J. [US/US]; Chicago, IL 60615 (US). VONSTEIN, Veronika [US/US]; Chicago, IL 60615 (US).</b>		(74) Agent: <b>CHAO, Mark; McDonnell Boenchen Hulbert &amp; Berghoff, 300 South Wacker Drive, Chicago, IL 60606 (US).</b> (81) Designated States: <b>AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GI, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).</b>  <b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>	
(54) Title: <b>METHOD FOR THE STABILIZATION OF PROTEINS AND THE THERMOSTABILIZED ALCOHOL DEHYDROGENASES PRODUCED THEREBY</b>			
(57) Abstract  The present invention provides a method for the directed evolution of proteins, particularly a method for improving the thermostability of proteins, particularly alcohol dehydrogenases, and especially horse liver alcohol dehydrogenase. The present invention also provides thermostabilized alcohol dehydrogenases produced according to this method.			

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METHOD FOR THE STABILIZATION OF PROTEINS AND THE  
THERMOSTABILIZED ALCOHOL DEHYDROGENASES PRODUCED THEREBY

TECHNICAL FIELD OF THE INVENTION

5       The present invention generally relates to a method  
for the directed evolution of proteins. In particular,  
the method is directed to stabilization of proteins such  
as dehydrogenases, and particularly is directed to a  
method for improving the thermostability of  
10   dehydrogenases such as alcohol dehydrogenases. The  
present invention also relates to thermostabilized  
alcohol dehydrogenases produced according to this method.

BACKGROUND OF THE INVENTION

15       Biocatalysts are enzymes which can specifically and  
efficiently expedite chemical reactions such as the  
synthesis of chemical compounds and biopolymers (Dixon et  
al., Enzymes (Academic Press, New York: 1979)).  
Biocatalysts are the key players in a number of important  
20   industrial synthetic and degradative applications  
including, but not limited to, the following:

- *Synthetic Applications* - Biocatalysts currently are  
employed as feasible alternatives to traditional  
catalysts, especially for the synthesis of chiral  
25   intermediates, or in the reduction of the number of  
protection/deprotection steps.
- *Biodegradation Applications* - Biocatalysts currently  
are employed as enzymatic degradation agents for  
environmental pollutants such as PCBs, chlorinated  
30   hydrocarbons, RDX, halogenated organic compounds,  
TNT, and other byproducts of industrial production  
that present significant health risks.
- *Diagnostics and Biosensors* - Biocatalysts currently  
are employed as detection agents in diagnostic tests  
35   and as biosensors which require enzyme durability.
- *Other large-scale industrial applications* -  
Biocatalysts currently are employed as catalysts in

the production of fuel supplies through conversion of agricultural feedstocks.

One enzyme that is of considerable utility in current enzymatic processes is the dehydrogenase. In particular, alcohol dehydrogenases are enzymes that command formal, reversible, two-electron chemistry in which alcohols are oxidized to the corresponding ketones. Depending on the precise reaction conditions, ketones can be reduced to the respective alcohols via a stereospecific delivery of a hydride equivalent catalyzed by the enzyme coupled to a bound cofactor such as NADH or NADPH (Lemiere, "Alcohol Dehydrogenase Catalyzed Oxidoreduction Reactions in Organic Chemistry", In Enzymes as Catalysts in Organic Synthesis, Schneider et al., Eds. (1986) p. 17). This system thus provides a mild, extremely sensitive route to chiral compounds, without contamination from undesired, competing reactions.

Such chiral compounds can be used, especially by the pharmaceutical industry, for the preparation of chiral therapeutics, and for effectively generating a wide variety of compounds having the capacity for industrial scale-up (Seebach et al., Org. Synth., 63, 1- (1984); Bradshaw et al., J. Org. Chem., 57, 1532 (1992); Hummel, Biotechnol. Lett., 12, 403 (1990)). In particular, dehydrogenases show promise for commercial application in the preparation of unusual amino acids and  $\beta$ -hydroxyketones, and in the resolution of racemic alcohols (Benoiton et al., J. Am. Chem. Soc., 79, 6192 (1957); Casy et al., Tetrahedron Lett., 33, 817 (1992); Jacovac et al., J. Am. Chem. Soc., 104, 4659-4665 (1982); Jones et al. Can. J. Chem., 60, 19 (1982)). Of the dehydrogenases, horse liver alcohol dehydrogenase (HLADH) is one of the most commonly used.

For an enzyme biocatalyst such as HLADH to prove useful in a wide-scale, practical, industrial

application, it is important that the biocatalyst possess the ability to survive harsh, dynamic, environmental and handling conditions inherent to large-scale commercial processes. These conditions include nonrefrigerated storage, and exposure to organic cosolvents and high reaction temperatures, as well as more idiosyncratic demands imposed by a particular industrial application.

To date, one of the greatest challenges associated with biocatalyst implementation is that of overcoming an overall intrinsic instability that results in a requirement for special preparative approaches and handling conditions. Many methods have been used in an attempt to stabilize certain proteins. Rational protein engineering has allowed the redesign of proteins with altered properties such as enhanced stability, shifted pH optima, and different substrate specificities (see, e.g., Bryan et al., Proteins, 1, 326-334 (1986); Pantoliano et al., Biochemistry, 26, 2077-82 (1987); Carter et al., Science, 237, 394-399 (1987); Wells et al., "Designing substrate specificity by protein engineering of electrostatic interactions",   , 84, 1219-1223 (1987); Grutter et al., Nature, 277, 667-669 (1979)).

While potentially an extremely powerful tool, rational protein engineering can be extremely time-consuming and expensive, and currently can be employed only for a very small number of enzymes having well-defined crystal or solution structures. Moreover, since the approach is tailored to a specific enzyme, it typically cannot be generalized to other enzyme species. Other post-production stabilization methods such as immobilization (Macaskie et al., FEMS Microbiol Rev., 14, 351-67 (1994); Shtelzer et al., Biotechnol. Appl. Biochem., 15, 227-35 (1992); Phadke, Biosystems, 27, 203-6 (1992)), or use of cross-linked enzymes (Navia et al., "Crosslinked enzyme crystals as robust biocatalysts", Proceedings of the Materials Research Society 1993 Symposium, Biomolecular Materials by Design (1993)),

suffer some of the same as well as further shortcomings, and similarly, are often too expensive to implement.

By contrast, directed evolution potentially can provide a practical approach to tailoring enzymes for a wide range of applications (Shao et al., "Engineering New Functions and Altering Existing Functions", Current Opinion in Structural Biology, in press (1996)). In support of this, enzymes have been shown to be highly adaptable molecules over evolutionary time scales. Many enzymes catalyzing very different reactions appear to have come about by divergent evolution, acquiring diverse capabilities by the processes of random mutation, recombination, and natural selection.

Thus, there remains a need for an effective means to randomly engineer better enzymes, particularly dehydrogenases, and especially, HLADH. The present invention seeks to overcome some of the aforesaid problems of enzyme design. In particular, it is an object of the present invention to provide a method for the directed evolution of enzymes, particularly dehydrogenases, and especially HLADH. It further is an object of the present invention to provide a method for stabilizing, e.g. improving the thermostability of enzymes such as dehydrogenases. Such a method of stabilizing dehydrogenases (particularly HLADH) would present a major advancement in the field since it would extend the shelf life, longevity, and active temperature range of these enzymes. These and other objects and advantages of the present invention, as well as further inventive features, will be apparent from the description of the invention provided herein.

#### BRIEF SUMMARY OF THE INVENTION

Briefly, the present invention provides, *inter alia*, a method for the stabilization of a protein (particularly for the stabilization of an alcohol dehydrogenase such as horse liver alcohol dehydrogenase (HLADH), general

enrichment/selection means that can be employed in *Escherichia* and *Thermus* to select for cells having altered levels of alcohol dehydrogenase activity as compared to a wild-type cell, thermostabilized HLADH proteins and nucleic acid sequences encoding same, as well as plasmids and hosts cells comprising the nucleic acid sequences.

#### BRIEF DESCRIPTION OF THE FIGURES

Figure 1 is a diagram that generally depicts the approach of the present invention for the accelerated evolution of enzymes. A pool of mutants of the particular gene is obtained by means such as spontaneous, directed, chemical, or PCR-mediated mutagenesis. The mutants of interest (i.e., having the particular stabilized feature) are identified by means of a screen or selection (A), and optionally, compatible mutations can be combined (e.g., by gene splicing, *in vitro* recombination, and the like) to enhance the stability even further (B).

Figure 2 is a digitized image of results of a filter assay for alcohol dehydrogenase activity which demonstrates that wild-type HLADH is rapidly inactivated at 75°C: no heat treatment (A); 5 minutes of heat treatment at 75°C (B); 10 minutes of heat treatment at 75°C (C); 15 minutes of heat treatment at 75°C (D); 20 minutes of heat treatment at 75°C (E); and 50 minutes of heat treatment at 75°C (F).

Figure 3 is a partial restriction map of the plasmid pTG450 which contains the *adh* gene from plasmid pBPP cloned into a pTG100kan<sup>tr2</sup> *Thermus* shuttle vector.

Figure 4 is a bar chart that depicts the increased thermostability of HLADH mutants produced according to the invention at 70°C. Cells containing pGEM-T (i.e., having no HLADH gene) did not show any HLADH activity.

Figure 5 is the sequence of *adh* gene [SEQ ID NO:1] that encodes the HLADH protein [SEQ ID NO:2], with the

location of certain mutations produced according to the invention identified as the boxed regions.

#### DETAILED DESCRIPTION OF THE INVENTION

5       The present invention provides, among other things, a method for stabilizing a certain feature of a protein (e.g., stability at a certain temperature, stability in the presence of certain reagents, etc.). In particular, the method of the invention provides a method for  
10   thermostabilizing a protein. Namely, the invention preferably provides a method of obtaining nonnative protein having a thermostability that is increased over that of the native version of said protein, as further described herein.

15       According to the invention, a "native" protein is the protein as it generally is found in nature. By contrast, a "nonnative" protein differs from the native protein in that it has been modified by human intervention, i.e., at either the level of the protein  
20   or its encoding DNA (e.g., by recombinant means to directly alter the genome; by unique selection and forced mutation; by random mutagenesis). Moreover, a "protein" desirably can be either an entire protein, or a portion of a protein (e.g., as where a chimeric  
25   nonnative protein results from either transcriptional or translational gene fusion). Similarly, a "nonnative protein" in some applications (e.g., applications for further study) may be a peptide (i.e., an incomplete protein), as where the peptide is chemically synthesized  
30   or, where a gene's coding sequence is transcribed or translated in vitro or, is produced by chemical processing of a complete protein.

      A preferred protein for stabilization, particularly thermostabilization according to the invention is a  
35   dehydrogenase, particularly an alcohol dehydrogenase, and especially horse liver alcohol dehydrogenase (e.g., as obtained from plasmid pBPP, and/or as set forth in



SEQ ID NO:2). Notably, with respect to SEQ ID NO:2, this protein does not initiate with methionine (Met). However, other variants of horse liver alcohol dehydrogenase produced by in vitro synthetic reactions, by means of chemical synthesis or, in other hosts (e.g., an eukaryotic host or other prokaryotic host cell) may possess a methionine residue in the first position of the protein. The numbering of residues in such proteins of course, would differ somewhat from that of SEQ ID NO:2. Namely, the second position of the aforementioned protein would be equivalent to the first position of the protein of SEQ ID NO:2. Of course, the ordinarily skilled artisan would know how to compare equivalent regions of proteins.

Desirably, other proteins (particularly proteins having capacity for industrial implementation) can be stabilized (e.g., thermostabilized) according to the invention. For instance, an alcohol dehydrogenase protein can be employed from another species. It is anticipated that this approach can be employed with alcohol dehydrogenases from other species based on the similarities between certain of the various alcohol dehydrogenases. Also, a protein according to the invention optionally can be another type of dehydrogenase, e.g., another type of NAD<sup>+</sup>(P)-linked dehydrogenase including, but not limited to, malate dehydrogenase, lactate dehydrogenase, isocitrate dehydrogenase (NADP<sup>+</sup>), hydroxylacyl CoA dehydrogenase, glyceraldehyde 3-phosphate dehydrogenase, and glucose 6-phosphate dehydrogenase (NADP<sup>+</sup>).

In a preferred embodiment, the method can be employed to thermostabilize a horse liver alcohol dehydrogenase. This method generally is depicted in Figure 1. Preferably the method comprises:

- (a) obtaining in a vector a gene that encodes the native protein;

(b) mutating the vector at more than one position in the gene to produce a vector library of cells comprising mutated versions of the gene;

5 (c) introducing the vector library *en masse* into cells of a strain in which the majority of the mutated versions of the gene are transcribed and translated to produce a cell library;

10 (d) screening the cell library to identify a cell comprising a mutated version of the gene that encodes a nonnative protein having a thermostability that is increased over that of the wild-type version of the protein; and

(e) purifying the cell from the cell library.

According to the invention, "gene that encodes said protein" can comprise a recombinant or nonrecombinant sequence, i.e., a sequence that is present as found in nature (i.e., encodes a native amino acid sequence) or, has been modified, for instance by the introduction of mutations (e.g., point mutations, insertions, deletions, or rearrangements) to comprise a nonnative amino acid sequence or, can be a mixture of native and nonnative amino acid sequences. Similarly, a recombinant gene may conjoin coding sequences (either in entirety or in part) with regulatory sequences (e.g., transcription initiation, transcription termination, translational start or stop sites, protein secretion sequences, and the like) which are not typically conjoined in nature. This can allow the production of a protein in a host in which it normally is not produced (e.g., production of a eukaryotic protein in a prokaryotic cell). Preferably, however, the recombinant gene (which can derive, in entirety or part, from any prokaryotic, eukaryotic, bacteriophage, or viral source) is capable of being transcribed and translated in a prokaryotic cell, particularly, a cell comprising a member of the genuses *Escherichi* or *Thermus*.

Thus, preferably a host cell in the context of the present invention (i.e., which can be employed in a method of stabilizing proteins) is a member of the kingdom Bacteria, Archaea, or Eukarya. In particular, preferably a cell employed in the method of stabilizing (particularly thermostabilizing) proteins according to the invention is a thermophile or hyperthermophile. In particular, preferably a cell is a member of the genus *Thermus*, and desirably is of the species *Thermus flavus*, *Thermus aquaticus*, *Thermus thermophilus*, or *Thermus sp.* Optimally a cell is either an *Escherichia coli* cell or a *Thermus aquaticus* cell.

The vector in which the gene of interest is subcloned can be any vector appropriate for delivery of a gene to a cell. For instance, the vector can be a plasmid, bacteriophage, virus, phagemid, cointegrate of one or more vector species, etc. Optimally, however, a vector is one that can be employed for gene expression in a prokaryotic cell such as a *Thermus* or *Escherichia* cell. It also is preferable that a vector have an ability to shuttle between different cells, e.g., between a *Thermus* and an *Escherichia* cell. One such vector that can be employed in the context of the invention is the vector pTG450.

The preferred method of the invention calls for mutating a vector containing the gene encoding the protein to be stabilized. Any method of mutagenesis such as is known to those skilled in the art and particularly as is described in the following Examples, can be employed in the method of the invention for generating a mutated gene. Desirably a PCR-based (error prone) approach, especially as set out as follows, is employed for mutagenesis. However, other mutagens (e.g., chemical mutagens such as hydroxylamine), also can be employed.

In the preferred method of mutagenesis employed in the invention, desirably the vector is mutated at more than one position in the gene of interest. This can be

assessed by means known in the art and as described in the Examples. Such mutagenesis in more than one position in the gene will result in a "vector library" comprising mutated versions of a gene, particularly of a horse  
5 liver alcohol dehydrogenase gene, which are present in the library mixture.

The vector library can be introduced *en masse* into cells (e.g., by transformation). Since the vectors and the cells employed for these methods are selected to be  
10 compatible, and the gene is engineered (e.g., as described below) to contain or to be flanked by any sequences necessary for its expression, it is expected that such introduction will result in the transcription and ensuing translation of the introduced gene.  
15 Moreover, such *en masse* introduction will result in the generation of a cell library comprising a mixture of cells transformed with plasmids having differing mutated genes. In some instances, it may be desirable to reisolate the vectors from the cell library (e.g., by a  
20 plasmid isolation or other vector isolation protocol), excise out the mutated gene, and subclone the mutated gene into another vector (e.g., a vector that has not been mutagenized).

Following the generation of the cell library, the  
25 cells preferably are screened under conditions that allow identification of a cell comprising a mutated version of the gene of interest that encodes a nonnative protein having a protein that is stabilized (e.g., thermostabilized) over that of the wild-type (i.e.,  
30 native) versions of the protein. A variety of selection means can be employed in accordance with the method of the present invention and, in particular, the selection means identified in the Examples which follow can be employed. Of course, one of ordinary skill in the art  
35 could modify these methods such that they are adapted for a particular host cell and/or a particular protein of interest. Desirably, however, screening conditions

are employed that provide for enrichment and/or selection for a cell containing nonnative DNA that encodes a protein having a particular feature of interest.

- 5        In particular, when the protein being stabilized according to the invention is an alcohol dehydrogenase, and particularly HLADH, the screen preferably can be carried out at increased temperature. For instance, desirably, screening is done at temperature a few  
10    degrees above and a few degrees below the temperature at which the native (i.e., wild-type) alcohol dehydrogenase is inactivated in the particular host cell employed for screening.

- According to this invention, "increasing the  
15    thermostability" of a nonnative protein means: (a) increasing the length of time at which a nonnative protein exhibits activity as compared to the wild-type protein; (b) increasing the temperature at which a nonnative protein exhibits activity as compared to a  
20    wild-type protein; or (c) increasing the length of time and temperature at which a nonnative protein exhibits activity as compared to a wild-type protein. A protein's activity can be determined by a variety of tests that differ with the various proteins to be tested. A few  
25    representative tests that can be employed in the method of the invention are set out in the following Examples. Preferably, however, "activity" means a detectable activity ranging from 10 to 90 units. For instance, whereas a wild-type protein might exhibit 10% activity  
30    at a defined temperature for a set amount of time, a thermostabilized enzyme might exhibit 10% activity at the same temperature for an increased amount of time, and/or might exhibit an activity at an increased temperature at which the native protein exhibits reduced  
35    or no activity.

The screening methods also desirably can be done, for instance, in the presence of alcohol, optionally at a lowered pH.

Following screening of cells to identify those  
5 having the desired trait(s) imparted by the mutated gene, optionally, cells exhibiting the trait can be further isolated. Vectors containing mutated versions of the gene of interest optionally can be further  
10 mutagenized by repeating steps (b) through (e) above to further stabilize the encoded protein.

The present invention accordingly also provides screens that can be employed to select for or against cells having altered ADH activity. For instance, the invention provides a method for selecting against growth  
15 of *Eschericia coli* recombinant cells which comprise levels of alcohol dehydrogenase that are higher than those of wild-type *Eschericia coli* cells. According to this invention, "growth" means an increase in cell mass, or some other evidence of cell metabolism such as one of  
20 ordinary skill in the art knows how to detect, or is described in the following Examples. An "absence of growth" means growth is not measurable by common procedures (e.g., visual or spectrophotometric observation and the like) or, cell killing. Cell killing  
25 can be determined by any well known means, e.g., visual observation, release of cell components, vital staining etc.

Thus the *E.coli* selection method comprises growing said recombinant cells under conditions selected from  
30 the group consisting of, wherein ethanol is present in a concentration of about 10%, isopropanol is present in a concentration of about 4%, and propanol is present in a concentration of about 2%, with the proviso that the wild-type cells exhibit reduced or an absence of growth  
35 under these conditions.

The present invention similarly provides a method for selecting for growth of *Thermus flavus* recombinant

cells which comprise levels of alcohol dehydrogenase that are higher than those of wild-type *Thermus flavus* cells. This method comprises growing the recombinant cells under conditions selected from the group consisting of wherein ethanol is present at a concentration of about 1% in a liquid or solid medium at a pH of about 7.0, with the proviso that the wild-type cells exhibit reduced or an absence of growth under these conditions.

As mentioned previously, these methods have been employed to thermostabilize HLADH. In particular, the invention provides an isolated and purified thermostabilized HLADH protein comprising a sequence selected from the group consisting of SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO:14, SEQ ID NO:16, SEQ ID NO:18 and SEQ ID NO:20. The invention also provides genes encoding such protein, e.g., an isolated and purified nucleic acid comprising a sequence selected from the group consisting of SEQ ID NO:3; SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:11, SEQ ID NO:13, SEQ ID NO:15, SEQ ID NO:17 and SEQ ID NO:19.

Moreover, the invention provides for plasmids encoding for such proteins: e.g., a plasmid comprising one of the aforementioned nucleic acid sequences; and a plasmid selected from the group consisting of pAD7; pAD8, pAD10, pAD91, pAD92, pAD93, pAD95, pAD111, pAD113, and pTG450.

The invention further preferably provides a method of increasing the thermostability of horse liver alcohol dehydrogenase. This method comprises introducing into a gene which encodes the alcohol dehydrogenase a mutation at a codon which codes for an amino acid residue at a position selected from the group consisting of the amino acid positions, 75, 94, 110, 177, 257, 268, 282, 292, and 297.

Examination of the three-dimensional structure of the HLADH protein will elucidate the manner in which further amino acid substitutions thermostabilizing the enzyme can be made, for instance, like-for-like (e.g., with acidic amino acids (i.e., aspartic acid, glutamic acid) being substituted for acidic amino acids; basic amino acids (i.e., lysine, arginine, histidine) being substituted for basic amino acids; sulfur containing amino acids (i.e., cysteine) being substituted for sulfur containing amino acids; amides (i.e., asparagine, glutamine) being substituted for amides, aliphatic nonpolar amino acids (i.e., glycine, alanine, valine, leucine, isoleucine) being substituted for aliphatic nonpolar amino acids; and alcoholic, aliphatic, and aromatic amino acids (i.e., serine, threonine, tyrosine, phenylalanine, and tryptophan) being substituted for alcoholic, aliphatic, and aromatic amino acids.

Additional uses and benefits of the invention will be apparent to one of ordinary skill in the art.

#### EXAMPLES

The following examples further illustrate the present invention but, of course, should not be construed as in any way limiting its scope.

#### EXAMPLE 1: Quantitative assay for ADH in cell extracts.

This example describes a method for the quantification of ADH in cell extracts, particularly for the quantitation of HLADH, that can be used according to the invention.

For this assay, overnight cultures of cells to be assayed are grown in rich media. The cells are washed, resuspended in 600  $\mu$ l of assay buffer (83 mM  $\text{KH}_2\text{PO}_4$  [pH 7.3], 40 mM KCl, 0.25 mM EDTA), and sonicated. The assay

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mixture contains 500  $\mu$ l of cell extract, 100  $\mu$ l EtOH, 20  $\mu$ l 100 mM NAD, 830  $\mu$ l buffer and is carried out at room temperature. The reaction is run for 3 minutes and absorbance at 340 nm is measured. Using this approach it is possible to identify a high IPTG inducible activity in the strains with the HLADH coding sequence under the control of the lacZ promoter. This method thus produces a reliable quantitative determination of HLADH activity present in the cell.

**EXAMPLE 2:** p-Rosaniline/alcohol plate screen in *E. coli*.

This example describes a plate screen for ADH activity that can be employed, for instance, in *E. coli*.

p-Rosaniline indicator plates are prepared according to Conway et al. (Conway et al., 169, 2591-2597 (1987)) by adding 8 ml of p-rosoaniline (2.5 mg/ml in 96% ethanol) and 100 mg of sodium bisulfite to 400 ml batches of precooled (45°C) Luria agar. Most of the dye is immediately converted to the leuco form by reaction with bisulfite to produce a rose-colored medium. Ethanol diffuses into the *E. coli* cells to produce the acetaldehyde by alcohol dehydrogenase. The leuco dye serves as a sink, reacting with the acetaldehyde to form a Schiff base which is intensely red. Thus, the plates can be streaked with a strain or, a strain can be applied in patches to the plate. Colonies will appear a deeper intensity of red dependent upon the level of ADH present in the cell. In particular, by plating appropriate controls on each plate, it is relatively easy to visually discern a strain which has a high level of dehydrogenase (deep red staining), an intermediate level of dehydrogenase (more moderate red staining), and no activity (little or no red staining).

This method thus provides a plate screen that can be employed in the method of the invention.

**EXAMPLE 3:** Filter screen for HLADH activity.

This example describes a sensitive plate assay of ADH activity which also allows colonies to be tested under different treatment conditions.

5 This assay relies for manipulation of bacterial colonies on the binding of the colonies to a nitrocellulose filter. The assay is carried out by a modified protocol described by Rellos et al. (Rellos et al., Protein Expression and Purification, 5, 270-277  
10 (1994)). Namely, a series of temperatures between 65 and 85°C in 5°C increments with incubation times varying from 10 minutes to one hour is analyzed in an attempt to determine the cutoff of the stability of the HLADH protein. For these experiments, the source of the *adh* gene encoding the HLADH enzyme was plasmid pBPP (Park et al., J. Biol. Chem., 266, 13296-13302 (1991)).

*E. coli* DH5 $\alpha$  cells containing plasmid pBPP (i.e., HLADH<sup>+</sup>) or plasmid pCRII (i.e., HLADH<sup>-</sup>) (Invitrogen; Carlsbad, CA) were grown on rich media plates at cell  
20 densities up to about 1,000 colonies per plate and transferred onto a nitrocellulose membrane. The adhered cells were lysed in Buffer 1 (10 mM KMes, pH 6.5, 0.5 mM CoCl<sub>2</sub>, 0.1% Triton X-100, 50  $\mu$ g/ml lysozyme, 10  $\mu$ g/ml DNase) in a chloroform bath for about one hour, washed  
25 once in Buffer 2 (10 mM KMes, 0.5 mM CoCl<sub>2</sub>, 0.2% BSA), and then washed two more times in Buffer 3 (Buffer 2 without BSA). The filters were then incubated at high temperatures in Buffer 4 (10 mM glycine, 0.5 mM CoCl<sub>2</sub>) and, after washing in Buffer 3, were incubated in the  
30 enzyme-detecting solution (30 mM Tris, pH 8.3, 2% ethanol, 1 mM NAD<sup>+</sup>, 0.1 mg/ml phenazine methosulfate, 1 mg/ml nitroblue tetrazolium) at room temperature for 3-5 minutes.

Results of these experiments are depicted in Figure  
35 2. As can be seen in this figure, the experiments confirm that a 15-20 minute treatment of the filters at 75°C resulted in roughly 90% inactivation of the HLADH

protein as estimated by the color changes. This information on the activity of the native protein can be used as a baseline for the identification and isolation of mutagenized candidates having altered ADH activity according to the invention.

**EXAMPLE 4:** Shuttle vectors and use of a p-rosaniline assay for verification of the activity of the HLADH gene in *Thermus*

In order to allow expression of the HLADH gene in both *Thermus* and *E. coli*, the gene was subcloned into the *Thermus* shuttle vector, pTG100kan<sup>tr2</sup> to create plasmid pTG450 depicted in Figure 3. In this construct, the gene is placed upstream of the thermostable kanamycin resistance gene (kan<sup>tr2</sup>), which is commanded by the lac promoter in *E. coli*, and the leu promoter in *Thermus*.

An *E. coli* strain harboring pTG450 has three times more HLADH activity in the presence of IPTG than the strain harboring the original pBPP plasmid. When transformed into *Thermus*, the *adh* gene integrates into the *leuB* site in the *Thermus* chromosome by a double recombination event. For these experiments, *Thermus flavus* was transformed with both the HLADH<sup>+</sup> plasmid pTG100kan<sup>tr2</sup> (i.e., creating strain TGF353) and the HLADH<sup>+</sup> plasmid TG450 (i.e., creating strain TGF650).

The presence of the *adh* gene in TGF650 was confirmed by PCR, and both TGF353 and TGF650 cells were assayed using a variation of the p-rosaniline plate assay described in Example 2. Namely, the agar overlay contained the same ingredients described, except TT media (Weber et al., Bio/Technology, 13, 271-275 (1995); Oshima et al., International Journal of Systematic Bacteriology, 24, 102-112 (1974)) was employed instead of Luria broth. A standard p-rosaniline plate can not be used since the indicator dye will spontaneously convert to the Schiff base if incubated overnight in the plate as part of this assay.

Using this approach, HLADH activity was observed in the pTG450 *Thermus* transformants at a level well above background levels observed for the pTG100kan<sup>tr2</sup> *Thermus* transformants. The activity was observed up to 70°C.

- 5 These results thus confirm that a *p*-rosaniline plate assay similarly can be employed in the context of the present invention for screening in *Thermus* for mutants having altered ADH activity.

- 10 EXAMPLE 5: Development of a Method of HLADH Selection/Enrichment in *E. coli*

This example describes a method of negative selection for growth of *E. coli* strains harboring the *adh* gene.

- 15 For these experiments, *E. coli* DH5 $\alpha$  cells containing either pTG100kan<sup>tr2</sup> (i.e., HLADH<sup>-</sup>) or pTG450 (i.e., HLADH<sup>+</sup>) were grown on LB plates with different alcohols in concentrations ranging from 2% to 12%. The results of one such experiment are displayed in Table 1.

**Table 1.** Effect of varying concentrations of alcohol in *Escherichia coli* strain DH5 $\alpha$  containing either containing or not containing a plasmid

	% of Methanol					% of Ethanol					% of Isopropanol					% of Propanol				
	2	4	8	10	12	2	4	8	10	12	2	4	6	8	12	2	4	8	12	
DH5 $\alpha$																				
PTG100kan <sup>rr</sup>	++	++	++	++	++	++	++	++	++	-	+	++	+-	-	-	++	-	-	-	-
PTG450	++	++	+	+	+-	++	++	+-	+-	-	+	+-	-	-	-	+-	-	-	-	-

Symbols in order of decreasing growth: ++, +, +-, -

As can be seen from Table 1, *E. coli* cells harboring high activity of HLADH (i.e., transformed with the HLADH' plasmid pTG450) are more sensitive to the presence of the alcohols in high concentrations. This probably is due to the accumulation of toxic aldehyde levels in the cells which result from the alcohol dehydrogenase reaction. Three other alcohols were tested (i.e., benzyl alcohol, hexyl alcohol, and hexyl amine), but did not give clear results because of their poor solubility in the media.

The experiment was repeated several times and the alcohol levels were refined to determine a range resulting in a clear selection. Three of the alcohols, i.e., ethanol at a concentration of 10%, isopropanol at a concentration of 4%, and propanol at a concentration of 2%, resulted in clean, negative selection for growth of *E. coli* harboring the *adh* gene.

These results thus confirm that the selection scheme can be employed for the isolation of mutants with altered ADH activity and, in particular, to select against *E. coli* strains having high levels of ADH. Such a system of negative selection also can be employed to affirmatively identify mutants having high levels of ADH. For instance, cells can be replica plated onto a series of plates from a single master plate prior to their transfer to nitrocellulose membranes. One of the plates can be retained, instead of being transferred to nitrocellulose, and matched against the sensitive cells identified in the assay. Cells of interest can then be recovered from the untreated plates.

**EXAMPLE 6:** Development of a Method of HLADH Selection/Enrichment in *Thermus*

This example describes the growth of *Thermus* strains in the presence of the high concentrations of

alcohols as a general method for selecting for growth of *Thermus* strains having high levels of ADH activity.

A series of experiments was conducted to develop a selection using alcohol levels in *Thermus*. In these  
5 experiments, *Thermus flavus* strains TGF353 (HLADH<sup>-</sup>) and TGF670 (HLADH<sup>+</sup>) were employed. Each strain was grown for two days on *Thermus* rich media (e.g., TT media, as described in Oshima et al., International Journal of Systematic Bacteriology, 24, 102-112 (1974)) present in  
10 plates or, was grown overnight in 4 ml of liquid TT medium, in order to ensure the cells were at the same physiological stage prior to testing. The test itself was performed on TT media and *Thermus* minimal media (Yeh et al., J. Biol. Chem., 251, 3134-3139 (1976) containing  
15 Casaminoacids (TMIN, CAA). Over a series of many experiments, the strains were grown on agar plates or in liquid medium containing various concentrations of ethanol (i.e., 0.5, 1, 2, 4, 6, or 8%), various concentrations of methanol (i.e., 2, 4, 6, or 8%),  
20 various concentrations of isopropanol (i.e., 0.5, 1, 2, 4, or 6%), various concentrations of propanol (i.e., 1, 2, 4, or 6%), or various concentrations of propanediol (i.e. 0.5 or 1%). Such experiments further were done at different pHs, i.e., at pH 7.0, 7.5 and 8.0, for the  
25 various alcohols at different concentrations. The results of one of these experiments is set out in Table 2.

Table 2. Optical density (OD<sub>600</sub>) in various media

Strain	pH 7.0			pH 7.0			pH 7.5			pH 7.5		
	% Ethanol			% Isopropanol			% Ethanol			% Isopropanol		
TGF353	--	0.5	1 2	0.5	1 2		--	0.5	1 2	0.5	1 2	
(ADH')	2.6	1.6	0.1 0.2	0.2	0.1 0.1		2.4	2.2	1.1 0.2	1.9	0.2 0.04	
TGF670	2.2	1.2	1.6 0.3	1.9	0.6 0.1		1.9	1.9	1.6 0.3	1.8	2.2 0.2	
(ADH')												



As can be seen from this experiment, the HLADH<sup>+</sup> strain TGF670 demonstrates higher resistance to alcohols than the HLADH<sup>-</sup> strain TGF353. Moreover, this selection  
5 appears to be dependent on pH, with the selection functioning better at lower pH, especially with ethanol. The selection thus may work by lowering the pH of the media--*Thermus* prefers higher pH for growth, in the range of pH 7.5-8.5 -- although not enough *Thermus*  
10 biochemistry is known to make this conclusive.

A similar effect can also be achieved on plates. However, the primary effect of the screen in *Thermus* is to retard growth of cells without the *adh* gene, not to completely eliminate it. This also is the case with the  
15 liquid media, indicating that a completely clean selection in *Thermus* without background is difficult to achieve. Nevertheless, this selection means provides a powerful enrichment, especially in liquid, by selecting for faster growing cells under the conditions defined.

20 The results thus confirm that the enrichment/selection means outlined above can be employed with *Thermus*.

**EXAMPLE 7:** Hydroxylamine mutagenesis of the *adh* gene.

25 This example describes mutagenesis of the *adh* gene as a representative alcohol dehydrogenase gene using the mutagen hydroxylamine (HA).

For HA mutagenesis of the *adh* gene, plasmids pBPP and pTG450, both of which contain this gene, were treated  
30 with HA using a standard approach. Namely, approximately 8 µg of plasmid DNA was mixed with 0.5 M NH<sub>2</sub>OH and incubated at 37°C for various lengths of time. For example, aliquots were taken at 1, 2, 3, or 4 hours following treatment, or following overnight exposure to  
35 the mutagen. The plasmid DNA was then transformed into *E. coli* strain DH5α and plated onto LB<sub>Ap100</sub> plates (i.e. LB plates containing 100 µg/ml ampicillin). Transformants

were analyzed by the ADH filter assay described in Example 3, and also using the p-rosaniline assay described in Example 2 to estimate the efficiency of mutagenesis.

- 5       After overnight treatment, only 3 - 4% plasmids treated with HA remained active. Plasmids treated by HA under conditions providing ~50% of inactivation of the *adh* were then transformed into *E. coli* strain NM554 (obtained from New England Biolabs) to obtain 500 - 700
- 10       transformant colonies per plate. These colonies were analyzed by the nitrocellulose filter ADH assay described in Example 3. For heat inactivation of ADH, the filters were incubated for 15 minutes at 70 °C in a hybridization oven.
- 15       Approximately 20,000 transformants were screened using this rapid method. Eighteen candidates were identified which appeared to show increased ADH thermotolerance. The candidates were purified and assayed on the same filter as control strains (i.e.,
- 20       strain XL1 containing the LADH<sup>+</sup> plasmid pBPP, and strain NM554 containing the LADH<sup>-</sup> plasmid pBluescript).

- Based on results of the filter screening, none of the identified candidates appeared to have the temperature-resistant phenotype suggested by the results
- 25       of the ADH filter assay. It is possible, however, that thermoresistant mutants can be obtained with HA upon further screening. Moreover, the chances of obtaining mutagenized *adh* resulting in enzyme thermostabilization might be further increased by excising the mutagenized
- 30       gene from the vector, and resubcloning into a wild-type vector (i.e., a vector that has not been treated with HA), followed by screening.

**EXAMPLE 8:**       PCR Mutagenesis of the *adh* gene

- 35       This example describes PCR mutagenesis of the *adh* gene as a representative alcohol dehydrogenase gene.

To increase the efficiency of the cloning of mutagenized *adh*, primers for directional cloning were employed:

CCC CGA ATT CTC AAA ACG TCA GGA TGG TAC G ADH(*EcoRI*) [SEQ

5 ID NO:21]

CCC CTC TAG AAT AAA TGA GCA CAG CAG GAA AAG TAA TAA AAT  
GC

ADH(*XbaI*) [SEQ ID NO:22]

The *adh* gene was amplified using these primers and cloned  
10 into a pGEM-T vector.

For PCR mutagenesis two protocols were used, one according to Spee et al. (Spee et al., Nucl. Acids Res., 21, 777-778 (1993)), and another according to Rellos et al., (Rellos et al., supra) in which the limiting dNTP  
15 concentration was double that of the first procedure and dITP was not employed. The pGEM-T plasmid containing the *adh* gene was then used as a template for PCR mutagenesis of *adh* using standard T7 and SP6 primers to perform the error-prone PCR reaction under these conditions.

20 Mutagenized *adh*-containing fragments were digested using *XbaI* and *EcoRI* enzymes, and subcloned into pBluescript SK to create a pBlue-ADH library. The resultant pBlue-ADH library (i.e., one library for each mutagenesis method performed) was transformed *en masse*  
25 into *E. coli* strain NM554 to allow the *adh* gene to be transcribed from the *lac* promoter. Transformants were then analyzed: (i) by PCR to determine the efficiency of cloning (% of the plasmids with and without insert), and  
30 ii) by ADH filter assay to determine the efficiency of mutagenesis (% inactive ADH clones). The results of these analyses are shown in Table 3.

Table 3. Mutant candidates identified

Method of mutagenesis*	Percentage of the plasmids with the insert	Percentage of the ADH <sup>+</sup> clones
Method No. 1	60%	64%
Method No. 2	90%	36%
No mutagenesis (wild-type <i>adh</i> )	80%	75%

\* Method No.1 was done according to Spee et al., supra,  
(i.e. with 14  $\mu$ M of limiting dNTP and 200  $\mu$ M dITP) and  
Method No. 2 was done according to Rellos et al., supra  
(i.e. without dITP and with 25  $\mu$ M of the limiting dNTP).

As can be seen from these results, both the cloning and mutagenesis efficiency was better using the second method.

The transformants were then plated to a density of 500 - 700 cells per plate and assayed on the filters under the same conditions described in the prior example for HA-mutagenesis of the *adh* gene. Approximately 5,000 clones containing *adh* mutagenized by the first method, and the same number of clones mutagenized by the second method, were tested. No thermostable candidates from the first method were identified. By contrast, thirteen candidates were selected from clones mutagenized by the second method which appeared to possess an HLADH variant that was more stable than the wild-type enzyme. Upon restreaking and retesting these colonies by the filter assay method, nine of the thirteen candidates (i.e., plasmids pAD7, pAD8, pAD10, pAD91, pAD92, pAD93, pAD95, pAD111, and pAD113) were chosen for further characterization.

These results confirm that PCR-mediated mutagenesis, particularly as described herein, can be employed to

obtain potential thermostable LADH variants. The results further indicate that the method can be employed to obtain other stabilized alcohol dehydrogenases, or other stabilized proteins.

5

**EXAMPLE 9:** Characterization of thermotolerant HLADH candidates.

This example describes a characterization for increased thermostability of mutants identified in the prior example.

10 These experiments were done by calculating the residual HLADH activity at 70°C for a series of incubation periods. Residual activity is calculated as activity after incubation at a particular temperature divided by activity before incubation. Cultures of the mutant candidates as well as control cells harboring the wild-type HLADH' control plasmid pBPP and HLADH' negative control plasmid pGEM-T were grown in appropriate media, and cell extracts were made by sonication. The extracts were then incubated at 70°C, taking an initial sample ( $t_0$ ), and sampling at about 30, 60, and 120 minutes. The samples were stored on ice, and the HLADH activity was determined spectrophotometrically as described in Example 1. The data was plotted as a percentage of activity compared to the  $t_0$  activity (residual activity) in order to compare the individual samples to each other and adjust for variations in expression levels or growth variations.

Figure 4 displays the residual activity data for the nine candidate plasmids pAD7, pAD8, pAD10, pAD91, pAD92, pAD93, pAD95, pAD111, and pAD113, wherein the  $t_0$  activity is normalized to 1.00 (100%). As can be seen from Figure 4, all the mutants exhibited increased thermotolerance compared to cells containing plasmid pBPP, which contains the wild-type HLADH gene. In particular, plasmids pAD91, pAD92, and pAD10 showed the most noticeable alterations

in thermostability. Cells containing pGEM-T (i.e., not having an HLADH gene) did not show any HLADH activity.

These results thus confirm that the method of the invention can be employed to obtain thermostable alcohol  
5 dehydrase, particularly HLADH, mutants.

Table 4 below provides data illustrating comparative data for HALDH activities in the original wild-type ("WT") clone and mutants. All clones were grown in 50 ml  
10 of LB medium with 100 µg/ml Amp (12.5 µg/ml Tet for WT clone) overnight, concentrated in 1 ml of the assay buffer (83 mM KH<sub>2</sub>PO<sub>4</sub>, 40 mM KCl, 0.25 mM EDTA), sonicated and assayed with ethanol as a substrate and NAD cofactor, with results shown as U = mol/mg protein x 1000 / percent  
15 residual activity.

Table 4. HALDH Activity after Heat Treatment

Strain	RT	Heat Treatment time		
		15 min	30 min	60 min
pADH7	8/100%	4/50%	2/25%	0.6/8%
pADH8	21/100%	7.4/35%	2/10%	0.2/1%
pADH10	16/100%	4/25%	1.4/9%	0/0%
pADH91	11/100%	8/73%	6/55%	4/36%
pADH92	25/100%	15/60%	17/68%	12/48%
pADH93	6/100%	1/17%	2.5/42%	0/0%
pADH95	66/100%	21/32%	10/15%	3/5%
pADH111	22/100%	15/68%	16/73%	11/50%
pADH113	9/100%	4/44%	3/33%	0.8/9%
WT	10/100%	1/10%	0.3/3%	0/0%

20 Table 5 below provides data illustrating comparative data for HALDH activities of the original wild-type ("WT") clone and mutants and substrate specificity. All clones were grown in 1 L of LB medium with 100 µg/ml Amp (12.5 µg/ml Tet for WT clone) overnight, concentrated in  
25 50 ml of the assay buffer (83 mM KH<sub>2</sub>PO<sub>4</sub>, 40 mM KCl, 0.25 mM EDTA), sonicated, incubated at 55°C for 5 min to denature the *E.coli* proteins and lyophilized. The assays were performed at room temperature with the listed substrate and NAD cofactor, with results shown as U =  
30 mol/mg protein x 1000.

Table 5 HLADH Substrate Specificity

Strain	Ethanol	Isopropanol	Butanol	Benzyl Alcol
pADH7	8.7	0	5.3	1.4
pADH8	18.2	1.4	11	7
pADH10	15.6	3	11.5	4.7
pADH91	13.2	1.1	4.7	3.4
pADH92	23.5	2.3	11	6.8
pADH93	5.6	1	3	1.6
pADH95	48	0.7	21.3	4.5
pADH111	22.6	1.6	9.8	3
pADH113	7	1.1	3	5
WT	9.2	1.7	7.6	3.5

Strain	Hexanol	Cyclohexanol	R-(-)Butanol	S-(+)Butanol
pADH7	4	3	0	0
pADH8	15	49	2.4	2.2
pADH10	15	69	10	4
pADH91	5.8	23	2.4	1.7
pADH92	10.6	50	2.3	2.4
pADH93	3.9	22	2	1.4
pADH95	21.3	16.5	0.5	0.8
pADH111	9.4	58	4	2.7
pADH113	2.7	14.7	2	1.3
WT	10	42	4.3	2.9

5

**EXAMPLE 10:** Sequence Analysis of HLADH  
Thermotolerant Candidates

This examples describes the sequencing of the  
10 mutagenized *adh* genes.

The inserts of plasmids containing the mutagenized  
*adh* gene were sequenced using an ABI DNA sequencer, and  
compared to the sequence of the wild type protein. The  
translated nucleic acid/amino acid sequence for plasmids  
15 having the wild-type or mutant *adh* genes is given in  
**Figure 5**, with the positions of the non-silent mutations  
(i.e., those that change the encoded amino acid)  
indicated by the boxes. **Table 6** summarizes all the  
nucleic acid mutations and the respective amino acid  
20 changes, if any, introduced by the mutations.

Table 6. Mutations identified in thermotolerant candidates

Mutant plasmid	Base pair position	Amino acid position <sup>1</sup>	Original codon	Mutant codon	Amino acid change <sup>2</sup>
pAD7	774	257	ATG	ATA	Met257Ile
	878	292	GTG	GCG	Val292Ala
pAD8	285	94	ACT	ACC	no aa change
	806	268	GTC	GCC	Val268Ala
pAD10	227	75	AGC	AAC	Ser75Asn
pAD91/92	284	94	ACT	ATT	Thr94Ile
pAD93	847	282	TGT	AGT	Cys282Ser
	893	297	GAT	GGT	Asp297Gly
pAD95	774	257	ATG	ATA	Met257Ile
	878	292	GTG	GCG	Val292Ala
pAD111	532	177	TCT	ACT	Ser177Thr
pAD113	129	42	GCC	GCT	no aa change
	159	52	GTG	GTA	no aa change
	331	110	TTC	CTC	Phe110Leu

Also, the individual sequences of the mutant *adh* sequences are set forth in the Sequence Listing for pAD7 (i.e., nucleic acid sequence at SEQ ID NO:3 and amino acid sequence at SEQ ID NO:4), pAD8 (i.e., nucleic acid sequence at SEQ ID NO:5 and amino acid sequence at SEQ ID NO:6), pAD10 (i.e., nucleic acid sequence at SEQ ID NO:7 and amino acid sequence at SEQ ID NO:8), pAD91/pAD92 (i.e., nucleic acid sequence at SEQ ID NO:9 and amino acid sequence at SEQ ID NO:10), pAD93 (i.e., nucleic acid sequence at SEQ ID NO:11 and amino acid sequence at SEQ ID NO:12), pAD95 (i.e., nucleic acid sequence at SEQ ID NO:13 and amino acid sequence at SEQ ID NO:14), pAD111



(i.e., nucleic acid sequence at SEQ ID NO:15 and amino acid sequence at SEQ ID NO:16), and pAD113(i.e., nucleic acid sequence at SEQ ID NO:17 and amino acid sequence at SEQ ID NO:18).

5       The first numbered amino acid in the wild-type and mutant sequences is serine since, in the sequences studied, the initial methionine (Met) is not present in the final protein. However, it is possible that Met is present in the wild-type (or mutant) HLADH sequences that  
10       are produced in a different host, e.g., in a eukaryotic host, or when transcribed and translated from a different plasmid construct or chromosome.

      As can be seen from this data, the sequences of pAD91 and pAD92 are identical, which indicates the clones  
15       from which the DNA was isolated likely are siblings. Mutants containing plasmids pAD91, pAD92, pAD93, and pAD95 were identified from the same filter and mutants containing plasmids pAD111 and pAD113 were identified from the same filter assay. Also, in both pAD8 and  
20       pAD91/92, the coding sequence specifying amino acid 94 is mutated. Whereas this results in no change in this position in pAD8, a mutation is introduced here in pAD91/92. Similarly, two mutations in pAD113 are silent and do not produce an amino acid change. These silent  
25       mutations likely do not contribute substantially to the thermostability of the protein.

**EXAMPLE 11:**       Further thermostabilization  
                          of HLADH proteins

30       This example describes the means by which the thermostable proteins identified and characterized as in the prior examples can be further thermostabilized.

      Using the new mutants as a starting point, the process applied here can be reiterated to increase the  
35       thermostability of the HLADH enzyme even further. Namely, it is expected that combinations of the identified HLADH mutations or, combinations of these

mutations with other HLADH mutations, can further thermostabilize the enzyme.

In order to do this, the new thermoinactivation limits need to be defined as described in Example 3.

- 5 This is followed by a new round of mutagenesis performed as described in Examples 8, 9, and 10. In addition, the identified mutations can be put together in differing combinations by *in vitro* site-directed mutagenesis and further molecular biology methods (see, e.g., Sambrook et al., Molecular Cloning: A Laboratory Manual (Cold Spring Harbor Laboratory Press, NY, 1989)) that include DNA shuffling via PCR methods (Stemmer et al., Proc. Natl. Acad. Sci., 91, 10747-10751 (1994a); Stemmer et al., Nature, 340, 389-391 (1994b)). As they have done in the past, these methods are all expected to give further increases in the levels of thermostability of the enzyme or, in another similarly screened-for trait.

- 15 All of the references cited herein, including patents, patent applications, sequences, and publications, are hereby incorporated in their entireties by reference.

- 20 While this invention has been described with an emphasis upon preferred embodiments, it will be obvious to those of ordinary skill in the art that variations in the preferred embodiments can be used, including variations due to improvements in the art, and that the invention can be practiced otherwise than as specifically described herein. Accordingly, this invention includes all modifications encompassed within the spirit and scope of the invention as defined by the following claims.

## SEQUENCE LISTING

- (1) GENERAL INFORMATION:
- (i) APPLICANT: DAVID C. DEMIRJIAN  
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- (ii) TITLE OF INVENTION: Method For The Stabilization Of Proteins And The  
Thermostabilized Alcohol Dehydrogenases Produced Thereby
- (iii) NUMBER OF SEQUENCES: 24
- (iv) CORRESPONDENCE ADDRESS:  
(A) ADDRESSEE: McDonald Boehnen Hulbert & Berghoff  
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(F) ZIP: 60606
- (v) COMPUTER READABLE FORM:  
(A) MEDIUM TYPE: Floppy disk  
(B) COMPUTER: IBM PC compatible  
(C) OPERATING SYSTEM: PC-DOS/MS-DOS  
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30
- (vi) CURRENT APPLICATION DATA:  
(A) APPLICATION NUMBER:  
(B) FILING DATE:  
(C) CLASSIFICATION:
- (2) INFORMATION FOR SEQ ID NO:1:
- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 1128 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: double  
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:
- |   |     |
|---|-----|
| ATG AGC ACA GCA GGA AAA GTA ATA AAA TGC AAA GCG GCT GTG CTG TGG | 48  |
| Ser Thr Ala Gly Lys Val Ile Lys Cys Lys Ala Ala Val Leu Trp     | 15  |
| GAG GAA AAG AAA CCA TTT TCC ATC GAG GAG GTG GAG GTT GCA CCC CCG | 96  |
| Glu Glu Lys Lys Pro Phe Ser Ile Glu Glu Val Glu Val Ala Pro Pro | 30  |
| AAG GCC CAT GAA GTC CGT ATA AAG ATG GTG GCC ACA GGA ATT TGT CGC | 144 |
| Lys Ala His Glu Val Arg Ile Lys Met Val Ala Thr Gly Ile Cys Arg | 45  |
| TCA GAT GAC CAC GTG GTT AGT GGA ACC CTT GTC ACA CCT CTT CCT GTG | 192 |
| Ser Asp Asp His Val Val Ser Gly Thr Leu Val Thr Pro Leu Pro Val | 60  |
| ATC GCA GGC CAT GAG GCA GCG GGC ATT GTG GAG AGC ATT GGA GAA GGC | 240 |
| Ile Ala Gly His Glu Ala Ala Gly Ile Val Glu Ser Ile Gly Glu Gly | 75  |
| GTC ACT ACA GTA AGA CCA GGT GAT AAA GTC ATC CCA CTC TTT ACT CCC | 288 |
| Val Thr Thr Val Arg Pro Gly Asp Lys Val Ile Pro Leu Phe Thr Pro | 95  |
| CAG TGT GGA AAA TGC AGG GTT TGT AAG CAC CCT GAA GGC AAC TTC TGC | 336 |
| Gln Cys Gly Lys Cys Arg Val Cys Lys His Pro Glu Gly Asn Phe Cys | 110 |
| TTG AAA AAT GAT CTG AGC ATG CCT CGG GGA ACC ATG CAG GAT GGT ACC | 384 |
| Leu Lys Asn Asp Leu Ser Met Pro Arg Gly Thr Met Gln Asp Gly Thr | 125 |
| AGC AGG TTC ACC TGC AGA GGG AAG CCC ATC CAC CAC TTC CTT GGC ACC | 432 |
| Ser Arg Phe Thr Cys Arg Gly Lys Pro Ile His His Phe Leu Gly Thr | 140 |

34

AGC ACC TTC TCC CAG TAC ACC GTG GTG GAC GAG ATC TCA GTG GCC AAG 480  
 Ser Thr Phe Ser Gln Tyr Thr Val Val Asp Glu Ile Ser Val Ala Lys  
 145 150 155

5 ATC GAT GCG GCC TCA CCG CTG GAG AAA GTC TGT CTC ATT GGC TGT GGA 528  
 Ile Asp Ala Ala Ser Pro Leu Glu Lys Val Cys Leu Ile Gly Cys Gly  
 160 165 170 175

10 TTT TCT ACT GGT TAT GGG TCT GCA GTC AAG GTT GCC AAG GTC ACC CAG 576  
 Phe Ser Thr Gly Tyr Gly Ser Ala Val Lys Val Ala Lys Val Thr Gln  
 180 185 190

15 GGC TCC ACC TGT GCC GTG TTT GGC CTT GGA GGA GTG GGC CTG TCT GTT 624  
 Gly Ser Thr Cys Ala Val Phe Gly Leu Gly Gly Val Gly Leu Ser Val  
 195 200 205

20 ATC ATG GGC TGT AAA GCA GCC GGA GCG GCC AGG ATC ATT GGG GTG GAC 672  
 Ile Met Gly Cys Lys Ala Ala Gly Ala Ala Arg Ile Ile Gly Val Asp  
 210 215 220

ATC AAC AAA GAC AAG TTT GCA AAG GCC AAA GAA GTG GGT GCC ACT GAG 720  
 Ile Asn Lys Asp Lys Phe Ala Lys Ala Lys Glu Val Gly Ala Thr Glu  
 225 230 235

25 TGT GTC AAC CCT CAG GAC TAC AAG AAA CCC ATC CAG GAG GTG CTG ACA 768  
 Cys Val Asn Pro Gln Asp Tyr Lys Lys Pro Ile Gln Glu Val Leu Thr  
 240 245 250 255

30 GAA ATG AGC AAT GGA GGT GTG GAT TTT TCC TTT GAA GTC ATT GGT CCG 816  
 Glu Met Ser Asn Gly Gly Val Asp Phe Ser Phe Glu Val Ile Gly Arg  
 260 265 270

35 CTC GAC ACT ATG GTG ACT GCC TTG TCA TGC TGT CAA GAA GCA TAT GGT 864  
 Leu Asp Thr Met Val Thr Ala Leu Ser Cys Cys Gln Glu Ala Tyr Gly  
 275 280 285

40 GTG AGC GTC ATT GTG GGA GTA CCT CCT GAT TCC CAA AAT CTC TCT ATG 912  
 Val Ser Val Ile Val Gly Val Pro Pro Asp Ser Gln Asn Leu Ser Met  
 290 295 300

AAT CCT ATG TTG CTA CTG AGT GGA CGT ACC TGG AAA GGA GCT ATT TTT 960  
 Asn Pro Met Leu Leu Leu Ser Gly Arg Thr Trp Lys Gly Ala Ile Phe  
 305 310 315

45 GGC GGT TTT AAG AGT AAA GAT TCT GTC CCC AAA CTT GTG GCC GAT TTT 1008  
 Gly Gly Phe Lys Ser Lys Asp Ser Val Pro Lys Leu Val Ala Asp Phe  
 320 325 330 335

50 ATG GCT AAA AAG TTT GCA CTG GAT CCT TTA ATC ACC CAT GTT TTA CCT 1056  
 Met Ala Lys Lys Phe Ala Leu Asp Pro Leu Ile Thr His Val Leu Pro  
 340 345 350

55 TTT GAA AAA ATA AAT GAA GGA TTT GAC CTG CTT CGC TCT GGA GAG AGT 1104  
 Phe Glu Lys Ile Asn Glu Gly Phe Asp Leu Leu Arg Ser Gly Glu Ser  
 355 360 365

60 ATC CGT ACC ATC CTG ACG TTT TGA 1128  
 Ile Arg Thr Ile Leu Thr Phe  
 370

## (2) INFORMATION FOR SEQ ID NO:2:

65 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 374 amino acids  
 (B) TYPE: amino acid  
 (D) TOPOLOGY: linear

70 (ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

75 Ser Thr Ala Gly Lys Val Ile Lys Cys Lys Ala Ala Val Leu Trp Glu  
 1 5 10 15  
 Glu Lys Lys Pro Phe Ser Ile Glu Glu Val Glu Val Ala Pro Pro Lys  
 20 25 30  
 80 Ala His Glu Val Arg Ile Lys Met Val Ala Thr Gly Ile Cys Arg Ser  
 35 40 45  
 Asp Asp His Val Val Ser Gly Thr Leu Val Thr Pro Leu Pro Val Ile  
 50 55 60

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Ala Gly His Glu Ala Ala Gly Ile Val Glu Ser Ile Gly Glu Gly Val  
 65 70 75 80

5 Thr Thr Val Arg Pro Gly Asp Lys Val Ile Pro Leu Phe Thr Pro Gln  
 85 90 95

Cys Gly Lys Cys Arg Val Cys Lys His Pro Glu Gly Asn Phe Cys Leu  
 100 105 110

10 Lys Asn Asp Leu Ser Met Pro Arg Gly Thr Met Gln Asp Gly Thr Ser  
 115 120 125

Arg Phe Thr Cys Arg Gly Lys Pro Ile His His Phe Leu Gly Thr Ser  
 130 135 140

15 Thr Phe Ser Gln Tyr Thr Val Val Asp Glu Ile Ser Val Ala Lys Ile  
 145 150 155 160

20 Asp Ala Ala Ser Pro Leu Glu Lys Val Cys Leu Ile Gly Cys Gly Phe  
 165 170 175

Ser Thr Gly Tyr Gly Ser Ala Val Lys Val Ala Lys Val Thr Gln Gly  
 180 185 190

25 Ser Thr Cys Ala Val Phe Gly Leu Gly Gly Val Gly Leu Ser Val Ile  
 195 200 205

Met Gly Cys Lys Ala Ala Gly Ala Ala Arg Ile Ile Gly Val Asp Ile  
 210 215 220

30 Asn Lys Asp Lys Phe Ala Lys Ala Lys Glu Val Gly Ala Thr Glu Cys  
 225 230 235 240

Val Asn Pro Gln Asp Tyr Lys Lys Pro Ile Gln Glu Val Leu Thr Glu  
 245 250 255

35 Met Ser Asn Gly Gly Val Asp Phe Ser Phe Glu Val Ile Gly Arg Leu  
 260 265 270

40 Asp Thr Met Val Thr Ala Leu Ser Cys Cys Gln Glu Ala Tyr Gly Val  
 275 280 285

Ser Val Ile Val Gly Val Pro Pro Asp Ser Gln Asn Leu Ser Met Asn  
 290 295 300

45 Pro Met Leu Leu Leu Ser Gly Arg Thr Trp Lys Gly Ala Ile Phe Gly  
 305 310 315 320

50 Gly Phe Lys Ser Lys Asp Ser Val Pro Lys Leu Val Ala Asp Phe Met  
 325 330 335

Ala Lys Lys Phe Ala Leu Asp Pro Leu Ile Thr His Val Leu Pro Phe  
 340 345 350

55 Glu Lys Ile Asn Glu Gly Phe Asp Leu Leu Arg Ser Gly Glu Ser Ile  
 355 360 365

Arg Thr Ile Leu Thr Phe  
 370

## (2) INFORMATION FOR SEQ ID NO:3:

65 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 1128 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: double  
 (D) TOPOLOGY: linear

70 (ii) MOLECULE TYPE: DNA (genomic)

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

75 ATG AGC ACA GCA CGA AAA GTA ATA AAA TGC AAA GCG GCT GTG CTG TGG 48  
 Ser Thr Ala Gly Lys Val Ile Lys Cys Lys Ala Ala Val Leu Trp  
 1 5 10 15

80 GAG GAA AAG AAA CCA TTT TCC ATC GAG GAG GTG GAG GTT GCA CCC CCG 96  
 Glu Glu Lys Lys Pro Phe Ser Ile Glu Glu Val Glu Val Ala Pro Pro  
 20 25 30

AAG GCC CAT GAA GTC CGT ATA AAG ATG GTG GCC ACA GGA ATT TGT CGC 144  
 Lys Ala His Glu Val Arg Ile Lys Met Val Ala Thr Gly Ile Cys Arg

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	35	40	45	
5	TCA GAT GAC CAC GTG GTT AGT GGA ACC CTT GTC ACA CCT CTT CCT GTG Ser Asp Asp His Val Val Ser Gly Thr Leu Val Thr Pro Leu Pro Val	192		
10	ATC GCA GGC CAT GAG GCA GCG GGC ATT GTG GAG ACC ATT GGA GAA GGC Ile Ala Gly His Glu Ala Ala Gly Ile Val Glu Ser Ile Gly Glu Gly	240		
15	GTC ACT ACA GTA AGA CCA GGT GAT AAA GTC ATC CCA CTC TTT ACT CCC Val Thr Thr Val Arg Pro Gly Asp Lys Val Ile Pro Leu Phe Thr Pro	288		
20	CAG TGT GGA AAA TGC AGG GTT TGT AAG CAC CCT GAA GGC AAC TTC TGC Gln Cys Gly Lys Cys Arg Val Cys Lys His Pro Glu Gly Asn Phe Cys	336		
25	TTG AAA AAT GAT CTG AGC ATG CCT CGG GGA ACC ATG CAG GAT GGT ACC Leu Lys Asn Asp Leu Ser Met Pro Arg Gly Thr Met Gln Asp Gly Thr	384		
30	AGC AGG TTC ACC TGC AGA GGG AAG CCC ATC CAC CAC TTC CTT GGC ACC Ser Arg Phe Thr Cys Arg Gly Lys Pro Ile His His Phe Leu Gly Thr	432		
35	AGC ACC TTC TCC CAG TAC ACC GTG GTG GAC GAG ATC TCA GTG GCC AAG Ser Thr Phe Ser Gln Tyr Thr Val Val Asp Glu Ile Ser Val Ala Lys	480		
40	ATC GAT GCG GCC TCA CCG CTG GAG AAA GTC TGT CTC ATT GGC TGT GGA Ile Asp Ala Ala Ser Pro Leu Glu Lys Val Cys Leu Ile Gly Cys Gly	528		
45	TTT TCT ACT GGT TAT GGG TCT GCA GTC AAG GTT GCC AAG GTC ACC CAG Phe Ser Thr Gly Tyr Gly Ser Ala Val Lys Val Ala Lys Val Thr Gln	576		
50	GGC TCC ACC TGT GCC GTG TTT GGC CTT GGA GGA GTG GGC CTG TCT GTT Gly Ser Thr Cys Ala Val Phe Gly Leu Gly Gly Val Gly Leu Ser Val	624		
55	ATC ATG GGC TGT AAA GCA GCC GGA GCG GCC AGG ATC ATT GGG GTG GAC Ile Met Gly Cys Lys Ala Ala Gly Ala Ala Arg Ile Ile Gly Val Asp	672		
60	ATC AAC AAA GAC AAG TTT GCA AAG GCC AAA GAA GTG GGT GCC ACT GAG Ile Asn Lys Asp Lys Phe Ala Lys Ala Lys Glu Val Gly Ala Thr Glu	720		
65	TGT GTC AAC CCT CAG GAC TAC AAG AAA CCC ATC CAG GAG GTG CTG ACA Cys Val Asn Pro Gln Asp Tyr Lys Lys Pro Ile Gln Glu Val Leu Thr	768		
70	GAA ATA AGC AAT GGA GGT GTG GAT TTT TCC TTT GAA GTC ATT GGT CGG Glu Ile Ser Asn Gly Gly Val Asp Phe Ser Phe Glu Val Ile Gly Arg	816		
75	CTC GAC ACT ATG GTG ACT GCC TTG TCA TGC TGT CAA GAA GCA TAT GGT Leu Asp Thr Met Val Thr Ala Leu Ser Cys Cys Gln Glu Ala Tyr Gly	864		
80	GTG AGC GTC ATT GCG GGA GTA CCT CCT GAT TCC CAA AAT CTC TCT ATG Val Ser Val Ile Ala Gly Val Pro Pro Asp Ser Gln Asn Leu Ser Met	912		
	AAT CCT ATG TTG CTA CTG AGT GGA CGT ACC TGG AAA GGA GCT ATT TTT Asn Pro Met Leu Leu Leu Ser Gly Arg Thr Trp Lys Gly Ala Ile Phe	960		
	GGC GGT TTT AAG AGT AAA GAT TCT GTC CCC AAA CTT GTG GCC GAT TTT Gly Gly Phe Lys Ser Lys Asp Ser Val Pro Lys Leu Val Ala Asp Phe	1008		
	ATG GCT AAA AAG TTT GCA CTG GAT CCT TTA ATC ACC CAT GTT TTA CCT Met Ala Lys Lys Phe Ala Leu Asp Pro Leu Ile Thr His Val Leu Pro	1056		
	TTT GAA AAA ATA AAT GAA GGA TTT GAC CTG CTT CGC TCT GGA GAG AGT Phe Glu Lys Ile Asn Glu Gly Phe Asp Leu Leu Arg Ser Gly Glu Ser	1104		
	ATC CGT ACC ATC CTG ACG TTT TGA Ile Arg Thr Ile Leu Thr Phe	1128		

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5 (2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 374 amino acids  
 (B) TYPE: amino acid  
 (D) TOPOLOGY: linear

10

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

15 Ser Thr Ala Gly Lys Val Ile Lys Cys Lys Ala Ala Val Leu Trp Glu  
 1 5 10 15  
 Glu Lys Lys Pro Phe Ser Ile Glu Glu Val Glu Val Ala Pro Pro Lys  
 20 20 25 30  
 Ala His Glu Val Arg Ile Lys Met Val Ala Thr Gly Ile Cys Arg Ser  
 35 40 45  
 25 Asp Asp His Val Val Ser Gly Thr Leu Val Thr Pro Leu Pro Val Ile  
 50 55 60  
 Ala Gly His Glu Ala Ala Gly Ile Val Glu Ser Ile Gly Glu Gly Val  
 65 70 75 80  
 30 Thr Thr Val Arg Pro Gly Asp Lys Val Ile Pro Leu Phe Thr Pro Gln  
 85 90 95  
 Cys Gly Lys Cys Arg Val Cys Lys His Pro Glu Gly Asn Phe Cys Leu  
 100 105 110  
 35 Lys Asn Asp Leu Ser Met Pro Arg Gly Thr Met Gln Asp Gly Thr Ser  
 115 120 125  
 40 Arg Phe Thr Cys Arg Gly Lys Pro Ile His His Phe Leu Gly Thr Ser  
 130 135 140  
 Thr Phe Ser Gln Tyr Thr Val Val Asp Glu Ile Ser Val Ala Lys Ile  
 145 150 155 160  
 45 Asp Ala Ala Ser Pro Leu Glu Lys Val Cys Leu Ile Gly Cys Gly Phe  
 165 170 175  
 Ser Thr Gly Tyr Gly Ser Ala Val Lys Val Ala Lys Val Thr Gln Gly  
 180 185 190  
 50 Ser Thr Cys Ala Val Phe Gly Leu Gly Gly Val Gly Leu Ser Val Ile  
 195 200 205  
 Met Gly Cys Lys Ala Ala Gly Ala Ala Arg Ile Ile Gly Val Asp Ile  
 210 215 220  
 55 Asn Lys Asp Lys Phe Ala Lys Ala Lys Glu Val Gly Ala Thr Glu Cys  
 225 230 235 240  
 60 Val Asn Pro Gln Asp Tyr Lys Lys Pro Ile Gln Glu Val Leu Thr Glu  
 245 250 255  
 Ile Ser Asn Gly Gly Val Asp Phe Ser Phe Glu Val Ile Gly Arg Leu  
 260 265 270  
 65 Asp Thr Met Val Thr Ala Leu Ser Cys Cys Gln Glu Ala Tyr Gly Val  
 275 280 285  
 70 Ser Val Ile Ala Gly Val Pro Pro Asp Ser Gln Asn Leu Ser Met Asn  
 290 295 300  
 Pro Met Leu Leu Leu Ser Gly Arg Thr Trp Lys Gly Ala Ile Phe Gly  
 305 310 315 320  
 75 Gly Phe Lys Ser Lys Asp Ser Val Pro Lys Leu Val Ala Asp Phe Met  
 325 330 335  
 Ala Lys Lys Phe Ala Leu Asp Pro Leu Ile Thr His Val Leu Pro Phe  
 340 345 350  
 80 Glu Lys Ile Asn Glu Gly Phe Asp Leu Leu Arg Ser Gly Glu Ser Ile  
 355 360 365  
 Arg Thr Ile Leu Thr Phe

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5	(2) INFORMATION FOR SEQ ID NO:5:	
	(i) SEQUENCE CHARACTERISTICS:	
	(A) LENGTH: 1128 base pairs	
	(B) TYPE: nucleic acid	
10	(C) STRANDEDNESS: double	
	(D) TOPOLOGY: linear	
	(ii) MOLECULE TYPE: DNA (genomic)	
15	(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:	
	ATG AGC ACA GCA GGA AAA GTA ATA AAA TGC AAA GCG GCT GTG CTG TGG 48	
20	Ser Thr Ala Gly Lys Val Ile Lys Cys Lys Ala Ala Val Leu Trp 15	
	GAG GAA AAG AAA CCA TTT TCC ATC GAG GAG GTG GAG GTT GCA CCC CCG 96	
	Glu Glu Lys Lys Pro Phe Ser Ile Glu Val Glu Val Ala Pro Pro 30	
25	AAG GCC CAT GAA GTC CGT ATA AAG ATG GTG GCC ACA GGA ATT TGT CGC 144	
	Lys Ala His Glu Val Arg Ile Lys Met Val Ala Thr Gly Ile Cys Arg 45	
30	TCA GAT GAC CAC GTG GTT AGT GGA ACC CTT GTC ACA CCT CTT CCT GTG 192	
	Ser Asp Asp His Val Val Ser Gly Thr Leu Val Thr Pro Leu Pro Val 60	
35	ATC GCA GGC CAT GAG GCA GCG GGC ATT GTG GAG AGC ATT GGA GAA GGC 240	
	Ile Ala Gly His Glu Ala Ala Gly Ile Val Glu Ser Ile Gly Glu Gly 75	
40	GTC ACT ACA GTA AGA CCA GGT GAT AAA GTC ATC CCA CTC TTT ACC CCC 288	
	Val Thr Thr Val Arg Pro Gly Asp Lys Val Ile Pro Leu Phe Thr Pro 95	
45	CAG TGT GGA AAA TGC AGG GTT TGT AAG CAC CCT GAA GGC AAC TTC TGC 336	
	Gln Cys Gly Lys Cys Arg Val Cys Lys His Pro Glu Gly Asn Phe Cys 110	
50	TTG AAA AAT GAT CTG AGC ATG CCT CGG GGA ACC ATG CAG GAT GGT ACC 384	
	Leu Lys Asn Asp Leu Ser Met Pro Arg Gly Thr Met Gln Asp Gly Thr 125	
55	AGC AGG TTC ACC TGC AGA GGG AAG CCC ATC CAC CAC TTC CTT GGC ACC 432	
	Ser Arg Phe Thr Cys Arg Gly Lys Pro Ile His His Phe Leu Gly Thr 140	
60	AGC ACC TTC TCC CAG TAC ACC GTG GTG GAC GAG ATC TCA GTG GCC AAG 480	
	Ser Thr Phe Ser Gln Tyr Thr Val Val Asp Glu Ile Ser Val Ala Lys 155	
65	ATC GAT GCG GCC TCA CCG CTG GAG AAA GTC TGT CTC ATT GGC TGT GGA 528	
	Ile Asp Ala Ala Ser Pro Leu Glu Lys Val Cys Leu Ile Gly Cys Gly 175	
70	TTT TCT ACT GGT TAT GGG TCT GCA GTC AAG GTT GCC AAG GTC ACC CAG 576	
	Phe Ser Thr Gly Tyr Gly Ser Ala Val Lys Val Ala Lys Val Thr Gln 190	
75	GGC TCC ACC TGT GCC GTG TTT GGC CTT GGA GGA GTG GGC CTG TCT GTT 624	
	Gly Ser Thr Cys Ala Val Phe Gly Leu Gly Gly Val Gly Leu Ser Val 205	
80	ATC ATG GGC TGT AAA GCA GCC GGA GCG GCC AGG ATC ATT GGG GTG GAC 672	
	Ile Met Gly Cys Lys Ala Ala Gly Ala Ala Arg Ile Ile Gly Val Asp 220	
	ATC AAC AAA GAC AAG TTT GCA AAG GCC AAA GAA GTG GGT GCC ACT GAG 720	
	Ile Asn Lys Asp Lys Phe Ala Lys Ala Lys Glu Val Gly Ala Thr Glu 235	
	TGT GTC AAC CCT CAG GAC TAC AAG AAA CCC ATC CAG GAG GTG CTG ACA 768	
	Cys Val Asn Pro Gln Asp Tyr Lys Lys Pro Ile Gln Glu Val Leu Thr 255	
	GAA ATG AGC AAT GGA GGT GTG GAT TTT TCC TTT GAA GCC ATT GGT CGG 816	
	Glu Met Ser Asn Gly Gly Val Asp Phe Ser Phe Glu Ala Ile Gly Arg 270	



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	CTC GAC ACT ATG GTG ACT GCC TTG TCA TGC TGT CAA GAA GCA TAT GGT Leu Asp Thr Met Val Thr Ala Leu Ser Cys Cys Gln Glu Ala Tyr Gly 275 280 285	864
5	GTG AGC GTC ATT GTG GGA GTA CCT CCT GAT TCC CAA AAT CTC TCT ATG Val Ser Val Ile Val Gly Val Pro Pro Asp Ser Gln Asn Leu Ser Met 290 295 300	912
10	AAT CCT ATG TTG CTA CTG AGT GGA CGT ACC TGG AAA GGA GCT ATT TTT Asn Pro Met Leu Leu Leu Ser Gly Arg Thr Trp Lys Gly Ala Ile Phe 305 310 315	960
15	GGC GGT TTT AAG AGT AAA GAT TCT GTC CCC AAA CTT GTG GCC GAT TTT Gly Gly Phe Lys Ser Lys Asp Ser Val Pro Lys Leu Val Ala Asp Phe 320 325 330 335	1008
20	ATG GCT AAA AAG TTT GCA CTG GAT CCT TTA ATC ACC CAT GTT TTA CCT Met Ala Lys Lys Phe Ala Leu Asp Pro Leu Ile Thr His Val Leu Pro 340 345 350	1056
25	TTT GAA AAA ATA AAT GAA GGA TTT GAC CTG CTT CGC TCT GGA GAG AGT Phe Glu Lys Ile Asn Glu Gly Phe Asp Leu Leu Arg Ser Gly Glu Ser 355 360 365	1104
30	ATC CGT ACC ATC CTG ACG TTT TGA Ile Arg Thr Ile Leu Thr Phe 370	1128
30	(2) INFORMATION FOR SEQ ID NO:6:	
	(i) SEQUENCE CHARACTERISTICS:	
	(A) LENGTH: 374 amino acids	
	(B) TYPE: amino acid	
	(D) TOPOLOGY: linear	
	(ii) MOLECULE TYPE: protein	
	(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:	
40	Ser Thr Ala Gly Lys Val Ile Lys Cys Lys Ala Ala Val Leu Trp Glu 1 5 10 15	
45	Glu Lys Lys Pro Phe Ser Ile Glu Glu Val Glu Val Ala Pro Pro Lys 20 25 30	
	Ala His Glu Val Arg Ile Lys Met Val Ala Thr Gly Ile Cys Arg Ser 35 40 45	
50	Asp Asp His Val Val Ser Gly Thr Leu Val Thr Pro Leu Pro Val Ile 50 55 60	
55	Ala Gly His Glu Ala Ala Gly Ile Val Glu Ser Ile Gly Glu Gly Val 65 70 75 80	
	Thr Thr Val Arg Pro Gly Asp Lys Val Ile Pro Leu Phe Thr Pro Gln 85 90 95	
60	Cys Gly Lys Cys Arg Val Cys Lys His Pro Glu Gly Asn Phe Cys Leu 100 105 110	
	Lys Asn Asp Leu Ser Met Pro Arg Gly Thr Met Gln Asp Gly Thr Ser 115 120 125	
65	Arg Phe Thr Cys Arg Gly Lys Pro Ile His His Phe Leu Gly Thr Ser 130 135 140	
70	Thr Phe Ser Gln Tyr Thr Val Val Asp Glu Ile Ser Val Ala Lys Ile 145 150 155 160	
	Asp Ala Ala Ser Pro Leu Glu Lys Val Cys Leu Ile Gly Cys Gly Phe 165 170 175	
75	Ser Thr Gly Tyr Gly Ser Ala Val Lys Val Ala Lys Val Thr Gln Gly 180 185 190	
	Ser Thr Cys Ala Val Phe Gly Leu Gly Gly Val Gly Leu Ser Val Ile 195 200 205	
80	Met Gly Cys Lys Ala Ala Gly Ala Ala Arg Ile Ile Gly Val Asp Ile 210 215 220	
	Asn Lys Asp Lys Phe Ala Lys Ala Lys Glu Val Gly Ala Thr Glu Cys 225 230 235 240	

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	Val Asn Pro Gln Asp Tyr Lys Lys Pro Ile Gln Glu Val Leu Thr Glu	
	245 250 255	
5	Met Ser Asn Gly Gly Val Asp Phe Ser Phe Glu Ala Ile Gly Arg Leu	
	260 265 270	
10	Asp Thr Met Val Thr Ala Leu Ser Cys Cys Gln Glu Ala Tyr Gly Val	
	275 280 285	
15	Ser Val Ile Val Gly Val Pro Pro Asp Ser Gln Asn Leu Ser Met Asn	
	290 295 300	
20	Pro Met Leu Leu Leu Ser Gly Arg Thr Trp Lys Gly Ala Ile Phe Gly	
	305 310 315 320	
25	Gly Phe Lys Ser Lys Asp Ser Val Pro Lys Leu Val Ala Asp Phe Met	
	325 330 335	
30	Ala Lys Lys Phe Ala Leu Asp Pro Leu Ile Thr His Val Leu Pro Phe	
	340 345 350	
35	Glu Lys Ile Asn Glu Gly Phe Asp Leu Leu Arg Ser Gly Glu Ser Ile	
	355 360 365	
40	Arg Thr Ile Leu Thr Phe	
	370	
45	(2) INFORMATION FOR SEQ ID NO:7:	
	(i) SEQUENCE CHARACTERISTICS:	
	(A) LENGTH: 1128 base pairs	
	(B) TYPE: nucleic acid	
	(C) STRANDEDNESS: double	
	(D) TOPOLOGY: linear	
50	(ii) MOLECULE TYPE: DNA (genomic)	
55	(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:	
60	ATG AGC ACA GCA GGA AAA GTA ATA AAA TGC AAA GCG GCT GTG CTG TGG	48
	Ser Thr Ala Gly Lys Val Ile Lys Cys Lys Ala Ala Val Leu Trp	
	1 5 10 15	
65	GAG GAA AAG AAA CCA TTT TCC ATC GAG GAG GTG GAG GTT GCA CCC CCG	96
	Glu Glu Lys Lys Pro Phe Ser Ile Glu Val Glu Val Ala Pro Pro	
	20 25 30	
70	AAG GCC CAT GAA GTC CGT ATA AAG ATG GTG GCC ACA GGA ATT TGT CGC	144
	Lys Ala His Glu Val Arg Ile Lys Met Val Ala Thr Gly Ile Cys Arg	
	35 40 45	
75	TCA GAT GAC CAC GTG GTT AGT GGA ACC CTT GTC ACA CCT CTT CCT GTG	192
	Ser Asp Asp His Val Val Ser Gly Thr Leu Val Thr Pro Leu Pro Val	
	50 55 60	
80	ATC GCA GGC CAT GAG GCA GCG GGC ATT GTG GAG AAC ATT GGA GAA GGC	240
	Ile Ala Gly His Glu Ala Ala Gly Ile Val Glu Asn Ile Gly Glu Gly	
	65 70 75	
85	GTC ACT ACA GTA AGA CCA GGT GAT AAA GTC ATC CCA CTC TTT ACT CCC	288
	Val Thr Thr Val Arg Pro Gly Asp Lys Val Ile Pro Leu Phe Thr Pro	
	80 85 90 95	
90	CAG TGT GGA AAA TGC AGG GTT TGT AAG CAC CCT GAA GGC AAC TTC TGC	336
	Gln Cys Gly Lys Cys Arg Val Cys Lys His Pro Glu Gly Asn Phe Cys	
	100 105 110	
95	TTG AAA AAT GAT CTG AGC ATG CCT CGG GGA ACC ATG CAG GAT GGT ACC	384
	Leu Lys Asn Asp Leu Ser Met Pro Arg Gly Thr Met Gln Asp Gly Thr	
	115 120 125	
100	AGC AGG TTC ACC TGC AGA GGG AAG CCC ATC CAC CAC TTC CTT GGC ACC	432
	Ser Arg Phe Thr Cys Arg Gly Lys Pro Ile His His Phe Leu Gly Thr	
	130 135 140	
105	AGC ACC TTC TCC CAG TAC ACC GTG GTG GAC GAG ATC TCA GTG GCC AAG	480
	Ser Thr Phe Ser Gln Tyr Thr Val Val Asp Glu Ile Ser Val Ala Lys	
	145 150 155	
110	ATC GAT GCG GCC TCA CCG CTG GAG AAA GTC TGT CTC ATT GGC TGT GGA	528
	Ile Asp Ala Ala Ser Pro Leu Glu Lys Val Cys Leu Ile Gly Cys Gly	

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160 165 170 175

5 TTT TCT ACT GGT TAT GGG TCT GCA GTC AAG GTT GCC AAG GTC ACC CAG 576  
Phe Ser Thr Gly Tyr Gly Ser Ala Val Lys Val Ala Lys Val Thr Gln  
180 185 190

GGC TCC ACC TGT GCC GTG TTT GGC CTT GGA GCA GTG GGC CTG TCT GTT 624  
Gly Ser Thr Cys Ala Val Phe Gly Leu Gly Gly Val Gly Leu Ser Val  
195 200 205

10 ATC ATG GGC TGT AAA GCA GCC GGA GCG GCC AGG ATC ATT GGG GTG GAC 672  
Ile Met Gly Cys Lys Ala Ala Gly Ala Ala Arg Ile Ile Gly Val Asp  
210 215 220

15 ATC AAC AAA GAC AAG TTT GCA AAG GCC AAA GAA GTG GGT GCC ACT GAG 720  
Ile Asn Lys Asp Lys Phe Ala Lys Ala Lys Glu Val Gly Ala Thr Glu  
225 230 235

20 TGT GTC AAC CCT CAG GAC TAC AAG AAA CCC ATC CAG GAG GTG CTG ACA 768  
Cys Val Asn Pro Gln Asp Tyr Lys Lys Pro Ile Gln Glu Val Leu Thr  
240 245 250 255

GAA ATG AGC AAT GGA GGT GTG GAT TTT TCC TTT GAA GTC ATT GGT CGG 816  
Glu Met Ser Asn Gly Gly Val Asp Phe Ser Phe Glu Val Ile Gly Arg  
260 265 270

25 CTC GAC ACT ATG GTG ACT GCC TTG TCA TGC TGT CAA GAA GCA TAT GGT 864  
Leu Asp Thr Met Val Thr Ala Leu Ser Cys Cys Gln Glu Ala Tyr Gly  
275 280 285

30 GTG AGC GTC ATT GTG GGA GTA CCT CCT GAT TCC CAA AAT CTC TCT ATG 912  
Val Ser Val Ile Val Gly Val Pro Pro Asp Ser Gln Asn Leu Ser Met  
290 295 300

35 AAT CCT ATG TTG CTA CTG AGT GGA CGT ACC TGG AAA GGA GCT ATT TTT 960  
Asn Pro Met Leu Leu Leu Ser Gly Arg Thr Trp Lys Gly Ala Ile Phe  
305 310 315

40 GGC GGT TTT AAG AGT AAA GAT TCT GTC CCC AAA CTT GTG GCC GAT TTT 1008  
Gly Gly Phe Lys Ser Lys Asp Ser Val Pro Lys Leu Val Ala Asp Phe  
320 325 330 335

45 ATG GCT AAA AAG TTT GCA CTG GAT CCT TTA ATC ACC CAT GTT TTA CCT 1056  
Met Ala Lys Lys Phe Ala Leu Asp Pro Leu Ile Thr His Val Leu Pro  
340 345 350

50 TTT GAA AAA ATA AAT GAA GGA TTT GAC CTG CTT CGC TCT GGA GAG AGT 1104  
Phe Glu Lys Ile Asn Glu Gly Phe Asp Leu Leu Arg Ser Gly Glu Ser  
355 360 365

ATC CGT ACC ATC CTG ACG TTT TGA 1128  
Ile Arg Thr Ile Leu Thr Phe  
370

55 (2) INFORMATION FOR SEQ ID NO:8:

60 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 374 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

65 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Ser Thr Ala Gly Lys Val Ile Lys Cys Lys Ala Ala Val Leu Trp Glu  
1 5 10 15

70 Glu Lys Lys Pro Phe Ser Ile Glu Glu Val Glu Val Ala Pro Pro Lys  
20 25 30

Ala His Glu Val Arg Ile Lys Met Val Ala Thr Gly Ile Cys Arg Ser  
35 40 45

75 Asp Asp His Val Val Ser Gly Thr Leu Val Thr Pro Leu Pro Val Ile  
50 55 60

80 Ala Gly His Glu Ala Ala Gly Ile Val Glu Asn Ile Gly Glu Gly Val  
65 70 75 80

Thr Thr Val Arg Pro Gly Asp Lys Val Ile Pro Leu Phe Thr Pro Gln  
85 90 95

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Cys Gly Lys Cys Arg Val Cys Lys His Pro Glu Gly Asn Phe Cys Leu  
 100 105 110  
 5 Lys Asn Asp Leu Ser Met Pro Arg Gly Thr Met Gln Asp Gly Thr Ser  
 115 120 125  
 Arg Phe Thr Cys Arg Gly Lys Pro Ile His His Phe Leu Gly Thr Ser  
 130 135 140  
 10 Thr Phe Ser Gln Tyr Thr Val Val Asp Glu Ile Ser Val Ala Lys Ile  
 145 150 155 160  
 Asp Ala Ala Ser Pro Leu Glu Lys Val Cys Leu Ile Gly Cys Gly Phe  
 165 170 175  
 15 Ser Thr Gly Tyr Gly Ser Ala Val Lys Val Ala Lys Val Thr Gln Gly  
 180 185 190  
 20 Ser Thr Cys Ala Val Phe Gly Leu Gly Gly Val Gly Leu Ser Val Ile  
 195 200 205  
 Met Gly Cys Lys Ala Ala Gly Ala Ala Arg Ile Ile Gly Val Asp Ile  
 210 215 220  
 25 Asn Lys Asp Lys Phe Ala Lys Ala Lys Glu Val Gly Ala Thr Glu Cys  
 225 230 235 240  
 Val Asn Pro Gln Asp Tyr Lys Lys Pro Ile Gln Glu Val Leu Thr Glu  
 245 250 255  
 30 Met Ser Asn Gly Gly Val Asp Phe Ser Phe Glu Val Ile Gly Arg Leu  
 260 265 270  
 35 Asp Thr Met Val Thr Ala Leu Ser Cys Cys Gln Glu Ala Tyr Gly Val  
 275 280 285  
 Ser Val Ile Val Gly Val Pro Pro Asp Ser Gln Asn Leu Ser Met Asn  
 290 295 300  
 40 Pro Met Leu Leu Leu Ser Gly Arg Thr Trp Lys Gly Ala Ile Phe Gly  
 305 310 315 320  
 Gly Phe Lys Ser Lys Asp Ser Val Pro Lys Leu Val Ala Asp Phe Met  
 325 330 335  
 45 Ala Lys Lys Phe Ala Leu Asp Pro Leu Ile Thr His Val Leu Pro Phe  
 340 345 350  
 50 Glu Lys Ile Asn Glu Gly Phe Asp Leu Leu Arg Ser Gly Glu Ser Ile  
 355 360 365  
 Arg Thr Ile Leu Thr Phe  
 370  
 55 (2) INFORMATION FOR SEQ ID NO:9:  
 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 1128 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: double  
 (D) TOPOLOGY: linear  
 60  
 (ii) MOLECULE TYPE: DNA (genomic)  
 65  
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:  
 70 ATG AGC ACA GCA GGA AAA GTA ATA AAA TGC AAA GCG GCT GTG CTG TGG 48  
 Ser Thr Ala Gly Lys Val Ile Lys Cys Lys Ala Ala Val Leu Trp  
 1 5 10 15  
 GAG GAA AAG AAA CCA TTT TCC ATC GAG GAG GTG GAG GTT GCA CCC CCG 96  
 Glu Glu Lys Lys Pro Phe Ser Ile Glu Glu Val Glu Val Ala Pro Pro  
 20 25 30  
 AAG GCC CAT GAA GTC CGT ATA AAG ATG GTG GCC ACA GGA ATT TGT CGC 144  
 Lys Ala His Glu Val Arg Ile Lys Met Val Ala Thr Gly Ile Cys Arg  
 35 40 45  
 80 TCA GAT GAC CAC GTG GTT AGT GGA ACC CTT GTC ACA CCT CTT CCT GTG 192  
 Ser Asp Asp His Val Val Ser Gly Thr Leu Val Thr Pro Leu Pro Val  
 50 55 60

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	ATC GCA GGC CAT GAG GCA GCG GGC ATT GTG GAG AGC ATT GGA GAA GGC Ile Ala Gly His Glu Ala Ala Gly Ile Val Glu Ser Ile Gly Glu Gly	240
5	GTC ACT ACA GTA AGA CCA GGT GAT AAA GTC ATC CCA CTC TTT ATT CCC Val Thr Thr Val Arg Pro Gly Asp Lys Val Ile Pro Leu Phe Ile Pro	288
10	CAG TGT GGA AAA TGC AGG GTT TGT AAG CAC CCT GAA GGC AAC TTC TGC Gln Cys Gly Lys Cys Arg Val Cys Lys His Pro Glu Gly Asn Phe Cys	336
15	TTG AAA AAT GAT CTG AGC ATG CCT CGG GGA ACC ATG CAG GAT GGT ACC Leu Lys Asn Asp Leu Ser Met Pro Arg Gly Thr Met Gln Asp Gly Thr	384
20	AGC AGG TTC ACC TGC AGA GGG AAG CCC ATC CAC CAC TTC CTT GGC ACC Ser Arg Phe Thr Cys Arg Gly Lys Pro Ile His His Phe Leu Gly Thr	432
25	AGC ACC TTC TCC CAG TAC ACC GTG GTG GAC GAG ATC TCA GTG GCC AAG Ser Thr Phe Ser Gln Tyr Thr Val Val Asp Glu Ile Ser Val Ala Lys	480
30	ATC GAT GCG GCC TCA CCG CTG GAG AAA GTC TGT CTC ATT GGC TGT GGA Ile Asp Ala Ala Ser Pro Leu Glu Lys Val Cys Leu Ile Gly Cys Gly	528
35	TTT TCT ACT GGT TAT GGG TCT GCA GTC AAG GTT GCC AAG GTC ACC CAG Phe Ser Thr Gly Tyr Gly Ser Ala Val Lys Val Ala Lys Val Thr Gln	576
40	GGC TCC ACC TGT GCC GTG TTT GGC CTT GGA GGA GTG GGC CTG TCT GTT Gly Ser Thr Cys Ala Val Phe Gly Leu Gly Gly Val Gly Leu Ser Val	624
45	ATC ATG GGC TGT AAA GCA GCC GGA GCG GCC AGG ATC ATT GGG GTG GAC Ile Met Gly Cys Lys Ala Ala Gly Ala Ala Arg Ile Ile Gly Val Asp	672
50	ATC AAC AAA GAC AAG TTT GCA AAG GCC AAA GAA GTG GGT GCC ACT GAG Ile Asn Lys Asp Lys Phe Ala Lys Ala Lys Glu Val Gly Ala Thr Glu	720
55	TGT GTC AAC CCT CAG GAC TAC AAG AAA CCC ATC CAG GAG GTG CTG ACA Cys Val Asn Pro Gln Asp Tyr Lys Lys Pro Ile Gln Glu Val Leu Thr	768
60	GAA ATG AGC AAT GGA GGT GTG GAT TTT TCC TTT GAA GTC ATT GGT CGG Glu Met Ser Asn Gly Gly Val Asp Phe Ser Phe Glu Val Ile Gly Arg	816
65	CTC GAC ACT ATG GTG ACT GCC TTG TCA TGC TGT CAA GAA GCA TAT GGT Leu Asp Thr Met Val Thr Ala Leu Ser Cys Cys Gln Glu Ala Tyr Gly	864
70	GTG AGC GTC ATT GTG GGA GTA CCT CCT GAT TCC CAA AAT CTC TCT ATG Val Ser Val Ile Val Gly Val Pro Pro Asp Ser Gln Asn Leu Ser Met	912
75	AAT CCT ATG TTG CTA CTG AGT GGA CGT ACC TGG AAA GGA GCT ATT TTT Asn Pro Met Leu Leu Leu Ser Gly Arg Thr Trp Lys Gly Ala Ile Phe	960
80	GGC GGT TTT AAG AGT AAA GAT TCT GTC CCC AAA CTT GTG GCC GAT TTT Gly Gly Phe Lys Ser Lys Asp Ser Val Pro Lys Leu Val Ala Asp Phe	1008
85	ATG GCT AAA AAG TTT GCA CTG GAT CCT TTA ATC ACC CAT GTT TTA CCT Met Ala Lys Lys Phe Ala Leu Asp Pro Leu Ile Thr His Val Leu Pro	1056
90	TTT GAA AAA ATA AAT GAA GGA TTT GAC CTG CTT CGC TCT GGA GAG AGT Phe Glu Lys Ile Asn Glu Gly Phe Asp Leu Leu Arg Ser Gly Glu Ser	1104
95	ATC CGT ACC ATC CTG ACG TTT TGA Ile Arg Thr Ile Leu Thr Phe	1128

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

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(A) LENGTH: 374 amino acids  
 (B) TYPE: amino acid  
 (D) TOPOLOGY: linear

5 (ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

10 Ser Thr Ala Gly Lys Val Ile Lys Cys Lys Ala Ala Val Leu Trp Glu  
 1 5 10 15  
 Glu Lys Lys Pro Phe Ser Ile Glu Glu Val Glu Val Ala Pro Pro Lys  
 20 25 30  
 15 Ala His Glu Val Arg Ile Lys Met Val Ala Thr Gly Ile Cys Arg Ser  
 35 40 45  
 Asp Asp His Val Val Ser Gly Thr Leu Val Thr Pro Leu Pro Val Ile  
 50 55 60  
 20 Ala Gly His Glu Ala Ala Gly Ile Val Glu Ser Ile Gly Glu Gly Val  
 65 70 75 80  
 Thr Thr Val Arg Pro Gly Asp Lys Val Ile Pro Leu Phe Ile Pro Gln  
 85 90 95  
 25 Cys Gly Lys Cys Arg Val Cys Lys His Pro Glu Gly Asn Phe Cys Leu  
 100 105 110  
 30 Lys Asn Asp Leu Ser Met Pro Arg Gly Thr Met Gln Asp Gly Thr Ser  
 115 120 125  
 Arg Phe Thr Cys Arg Gly Lys Pro Ile His His Phe Leu Gly Thr Ser  
 130 135 140  
 35 Thr Phe Ser Gln Tyr Thr Val Val Asp Glu Ile Ser Val Ala Lys Ile  
 145 150 155 160  
 40 Asp Ala Ala Ser Pro Leu Glu Lys Val Cys Leu Ile Gly Cys Gly Phe  
 165 170 175  
 Ser Thr Gly Tyr Gly Ser Ala Val Lys Val Ala Lys Val Thr Gln Gly  
 180 185 190  
 45 Ser Thr Cys Ala Val Phe Gly Leu Gly Gly Val Gly Leu Ser Val Ile  
 195 200 205  
 Met Gly Cys Lys Ala Ala Gly Ala Ala Arg Ile Ile Gly Val Asp Ile  
 210 215 220  
 50 Asn Lys Asp Lys Phe Ala Lys Ala Lys Glu Val Gly Ala Thr Glu Cys  
 225 230 235 240  
 Val Asn Pro Gln Asp Tyr Lys Lys Pro Ile Gln Glu Val Leu Thr Glu  
 245 250 255  
 55 Met Ser Asn Gly Gly Val Asp Phe Ser Phe Glu Val Ile Gly Arg Leu  
 260 265 270  
 60 Asp Thr Met Val Thr Ala Leu Ser Cys Cys Gln Glu Ala Tyr Gly Val  
 275 280 285  
 Ser Val Ile Val Gly Val Pro Pro Asp Ser Gln Asn Leu Ser Met Asn  
 290 295 300  
 65 Pro Met Leu Leu Leu Ser Gly Arg Thr Trp Lys Gly Ala Ile Phe Gly  
 305 310 315 320  
 70 Gly Phe Lys Ser Lys Asp Ser Val Pro Lys Leu Val Ala Asp Phe Met  
 325 330 335  
 Ala Lys Lys Phe Ala Leu Asp Pro Leu Ile Thr His Val Leu Pro Phe  
 340 345 350  
 75 Glu Lys Ile Asn Glu Gly Phe Asp Leu Leu Arg Ser Gly Glu Ser Ile  
 355 360 365  
 80 Arg Thr Ile Leu Thr Phe  
 370

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

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(A) LENGTH: 1128 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: double  
 (D) TOPOLOGY: linear

5

(ii) MOLECULE TYPE: DNA (genomic)

10

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

ATG AGC ACA GCA GGA AAA GTA ATA AAA TGC AAA GCG GCT GTG CTG TGG	48
Ser Thr Ala Gly Lys Val Ile Lys Cys Lys Ala Ala Val Leu Trp	
1 5 10 15	
GAG GAA AAG AAA CCA TTT TCC ATC GAG GAG GTG GAG GTT GCA CCC CCG	96
Glu Glu Lys Lys Pro Phe Ser Ile Glu Glu Val Glu Val Ala Pro Pro	
20 25 30	
AAG GCC CAT GAA GTC CGT ATA AAG ATG GTG GCC ACA GGA ATT TGT CGC	144
Lys Ala His Glu Val Arg Ile Lys Met Val Ala Thr Gly Ile Cys Arg	
35 40 45	
TCA GAT GAC CAC GTG GTT AGT GGA ACC CTT GTC ACA CCT CTT CCT GTG	192
Ser Asp Asp His Val Val Ser Gly Thr Leu Val Thr Pro Leu Pro Val	
50 55 60	
ATC GCA GGC CAT GAG GCA GCG GGC ATT GTG GAG AGC ATT GGA GAA GGC	240
Ile Ala Gly His Glu Ala Ala Gly Ile Val Glu Ser Ile Gly Glu Gly	
65 70 75	
GTC ACT ACA GTA AGA CCA GGT GAT AAA GTC ATC CCA CTC TTT ACT CCC	288
Val Thr Thr Val Arg Pro Gly Asp Lys Val Ile Pro Leu Phe Thr Pro	
80 85 90 95	
CAG TGT GGA AAA TGC AGG GTT TGT AAG CAC CCT GAA GGC AAC TTC TGC	336
Gln Cys Gly Lys Cys Arg Val Cys Lys His Pro Glu Gly Asn Phe Cys	
100 105 110	
TTG AAA AAT GAT CTG AGC ATG CCT CGG GGA ACC ATG CAG GAT GGT ACC	384
Leu Lys Asn Asp Leu Ser Met Pro Arg Gly Thr Met Gln Asp Gly Thr	
115 120 125	
AGC AGG TTC ACC TGC AGA GGG AAG CCC ATC CAC CAC TTC CTT GGC ACC	432
Ser Arg Phe Thr Cys Arg Gly Lys Pro Ile His His Phe Leu Gly Thr	
130 135 140	
AGC ACC TTC TCC CAG TAC ACC GTG GTG GAC GAG ATC TCA GTG GCC AAG	480
Ser Thr Phe Ser Gln Tyr Thr Val Val Asp Glu Ile Ser Val Ala Lys	
145 150 155	
ATC GAT GCG GCC TCA CCG CTG GAG AAA GTC TGT CTC ATT GGC TGT GGA	528
Ile Asp Ala Ala Ser Pro Leu Glu Lys Val Cys Leu Ile Gly Cys Gly	
160 165 170 175	
TTT TCT ACT GGT TAT GGG TCT GCA GTC AAG GTT GCC AAG GTC ACC CAG	576
Phe Ser Thr Gly Tyr Ser Ala Val Lys Val Ala Lys Val Thr Gln	
180 185 190	
GGC TCC ACC TGT GCC GTG TTT GGC CTT GGA GGA GTG GGC CTG TCT GTT	624
Gly Ser Thr Cys Ala Val Phe Gly Leu Gly Gly Val Gly Leu Ser Val	
195 200 205	
ATC ATG GGC TGT AAA GCA GCC GGA GCG GCC AGG ATC ATT GGG GTG GAC	672
Ile Met Gly Cys Lys Ala Ala Gly Ala Ala Arg Ile Ile Gly Val Asp	
210 215 220	
ATC AAC AAA GAC AAG TTT GCA AAG GCC AAA GAA GTG GGT GCC ACT GAG	720
Ile Asn Lys Asp Lys Phe Ala Lys Ala Lys Glu Val Gly Ala Thr Glu	
225 230 235	
TGT GTC AAC CCT CAG GAC TAC AAG AAA CCC ATC CAG GAG GTG CTG ACA	768
Cys Val Asn Pro Gln Asp Tyr Lys Lys Pro Ile Gln Glu Val Leu Thr	
240 245 250 255	
GAA ATG AGC AAT GGA GGT GTG GAT TTT TCC TTT GAA GTC ATT GGT CGG	816
Glu Met Ser Asn Gly Gly Val Asp Phe Ser Phe Glu Val Ile Gly Arg	
260 265 270	
CTC GAC ACT ATG GTG ACT GCC TTG TCA TGC AGT CAA GAA GCA TAT GGT	864
Leu Asp Thr Met Val Thr Ala Leu Ser Cys Ser Gln Glu Ala Tyr Gly	
275 280 285	
GTG AGC GTC ATT GTG GGA GTA CCT CCT GGT TCC CAA AAT CTC TCT ATG	912
Val Ser Val Ile Val Gly Val Pro Pro Gly Ser Gln Asn Leu Ser Met	

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	290	295	300	
5	AAT CCT ATG TTG CTA CTG AGT GGA CGT ACC TGG AAA GGA GCT ATT TTT Asn Pro Met Leu Leu Leu Ser Gly Arg Thr Trp Lys Gly Ala Ile Phe 305 310 315			960
10	GGC GGT TTT AAG AGT AAA GAT TCT GTC CCC AAA CTT GTG GCC GAT TTT Gly Gly Phe Lys Ser Lys Asp Ser Val Pro Lys Leu Val Ala Asp Phe 320 325 330 335			1008
15	ATG GCT AAA AAG TTT GCA CTG GAT CCT TTA ATC ACC CAT GTT TTA CCT Met Ala Lys Lys Phe Ala Leu Asp Pro Leu Ile Thr His Val Leu Pro 340 345 350			1056
20	TTT GAA AAA ATA AAT GAA GGA TTT GAC CTG CTT CGC TCT GGA GAG AGT Phe Glu Lys Ile Asn Glu Gly Phe Asp Leu Leu Arg Ser Gly Glu Ser 355 360 365			1104
25	ATC CGT ACC ATC CTG ACG TTT TGA Ile Arg Thr Ile Leu Thr Phe 370			1128
30	(2) INFORMATION FOR SEQ ID NO:12:			
35	(i) SEQUENCE CHARACTERISTICS:			
	(A) LENGTH: 374 amino acids			
	(B) TYPE: amino acid			
	(D) TOPOLOGY: linear			
40	(ii) MOLECULE TYPE: protein			
45	(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:			
50	Ser Thr Ala Gly Lys Val Ile Lys Cys Lys Ala Ala Val Leu Trp Glu 1 5 10 15			
55	Glu Lys Lys Pro Phe Ser Ile Glu Glu Val Glu Val Ala Pro Pro Lys 20 25 30			
60	Ala His Glu Val Arg Ile Lys Met Val Ala Thr Gly Ile Cys Arg Ser 35 40 45			
65	Asp Asp His Val Val Ser Gly Thr Leu Val Thr Pro Leu Pro Val Ile 50 55 60			
70	Ala Gly His Glu Ala Ala Gly Ile Val Glu Ser Ile Gly Glu Gly Val 65 70 75 80			
75	Thr Thr Val Arg Pro Gly Asp Lys Val Ile Pro Leu Phe Thr Pro Gln 85 90 95			
80	Cys Gly Lys Cys Arg Val Cys Lys His Pro Glu Gly Asn Phe Cys Leu 100 105 110			
85	Lys Asn Asp Leu Ser Met Pro Arg Gly Thr Met Gln Asp Gly Thr Ser 115 120 125			
90	Arg Phe Thr Cys Arg Gly Lys Pro Ile His His Phe Leu Gly Thr Ser 130 135 140			
95	Thr Phe Ser Gln Tyr Thr Val Val Asp Glu Ile Ser Val Ala Lys Ile 145 150 155 160			
100	Asp Ala Ala Ser Pro Leu Glu Lys Val Cys Leu Ile Gly Cys Gly Phe 165 170 175			
105	Ser Thr Gly Tyr Gly Ser Ala Val Lys Val Ala Lys Val Thr Cln Gly 180 185 190			
110	Ser Thr Cys Ala Val Phe Gly Leu Gly Gly Val Gly Leu Ser Val Ile 195 200 205			
115	Met Gly Cys Lys Ala Ala Gly Ala Ala Arg Ile Ile Gly Val Asp Ile 210 215 220			
120	Asn Lys Asp Lys Phe Ala Lys Ala Lys Glu Val Gly Ala Thr Glu Cys 225 230 235 240			
125	Val Asn Pro Gln Asp Tyr Lys Lys Pro Ile Gln Glu Val Leu Thr Glu 245 250 255			
130	Met Ser Asn Gly Gly Val Asp Phe Ser Phe Glu Val Ile Gly Arg Leu 260 265 270			

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	Asp Thr Met Val Thr Ala Leu Ser Cys Ser Gln Glu Ala Tyr Gly Val	
	275 280 285	
5	Ser Val Ile Val Gly Val Pro Pro Gly Ser Gln Asn Leu Ser Met Asn	
	290 295 300	
	Pro Met Leu Leu Leu Ser Gly Arg Thr Trp Lys Gly Ala Ile Phe Gly	
10	305 310 315 320	
	Gly Phe Lys Ser Lys Asp Ser Val Pro Lys Leu Val Ala Asp Phe Met	
	325 330 335	
15	Ala Lys Lys Phe Ala Leu Asp Pro Leu Ile Thr His Val Leu Pro Phe	
	340 345 350	
	Glu Lys Ile Asn Glu Gly Phe Asp Leu Leu Arg Ser Gly Glu Ser Ile	
	355 360 365	
20	Arg Thr Ile Leu Thr Phe	
	370	
25	(2) INFORMATION FOR SEQ ID NO:13:	
	(i) SEQUENCE CHARACTERISTICS:	
	(A) LENGTH: 1128 base pairs	
	(B) TYPE: nucleic acid	
30	(C) STRANDEDNESS: double	
	(D) TOPOLOGY: linear	
	(ii) MOLECULE TYPE: DNA (genomic)	
35	(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:	
	ATG AGC ACA GCA GGA AAA GTA ATA AAA TGC AAA GCG GCT GTG CTG TGG	48
	Ser Thr Ala Gly Lys Val Ile Lys Cys Lys Ala Ala Val Leu Trp	
40	1 5 10 15	
	GAG GAA AAG AAA CCA TTT TCC ATC GAG GAG GTG GAG GTT GCA CCC CCG	96
	Glu Glu Lys Lys Pro Phe Ser Ile Glu Glu Val Glu Val Ala Pro Pro	
	20 25 30	
45	AAG GCC CAT GAA GTC CGT ATA AAG ATG GTG GCC ACA GGA ATT TGT CGC	144
	Lys Ala His Glu Val Arg Ile Lys Met Val Ala Thr Gly Ile Cys Arg	
	35 40 45	
50	TCA GAT GAC CAC GTG GTT AGT GGA ACC CTT GTC ACA CCT CTT CCT GTG	192
	Ser Asp Asp His Val Val Ser Gly Thr Leu Val Thr Pro Leu Pro Val	
	50 55 60	
55	ATC GCA GGC CAT GAG GCA GCG GGC ATT GTG GAG AGC ATT GGA GAA GGC	240
	Ile Ala Gly His Glu Ala Ala Gly Ile Val Glu Ser Ile Gly Glu Gly	
	65 70 75	
60	GTC ACT ACA GTA AGA CCA GGT GAT AAA GTC ATC CCA CTC TTT ACT CCC	288
	Val Thr Thr Val Arg Pro Gly Asp Lys Val Ile Pro Leu Phe Thr Pro	
	80 85 90 95	
	CAG TGT GGA AAA TGC AGG GTT TGT AAG CAC CCT GAA GGC AAC TTC TGC	336
	Gln Cys Gly Lys Cys Arg Val Cys Lys His Pro Glu Gly Asn Phe Cys	
	100 105 110	
65	TTG AAA AAT GAT CTG AGC ATG CCT CGG GGA ACC ATG CAG GAT GGT ACC	384
	Leu Lys Asn Asp Leu Ser Met Pro Arg Gly Thr Met Gln Asp Gly Thr	
	115 120 125	
70	AGC AGG TTC ACC TGC AGA GGG AAG CCC ATC CAC CAC TTC CTT GGC ACC	432
	Ser Arg Phe Thr Cys Arg Gly Lys Pro Ile His His Phe Leu Gly Thr	
	130 135 140	
75	AGC ACC TTC TCC CAG TAC ACC GTG GTG GAC GAG ATC TCA GTG GCC AAG	480
	Ser Thr Phe Ser Gln Tyr Thr Val Val Asp Glu Ile Ser Val Ala Lys	
	145 150 155	
	ATC GAT GCG GCC TCA CCG CTG GAG AAA GTC TGT CTC ATT GGC TGT GGA	528
	Ile Asp Ala Ala Ser Pro Leu Glu Lys Val Cys Leu Ile Gly Cys Gly	
	160 165 170 175	
80	TTT TCT ACT GGT TAT GGG TCT GCA GTC AAG GTT GCC AAG GTC ACC CAG	576
	Phe Ser Thr Gly Tyr Gly Ser Ala Val Lys Val Ala Lys Val Thr Gln	
	180 185 190	

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	GGC TCC ACC TGT GCC GTG TTT GGC CTT GGA GGA GTG GGC CTG TCT GTT	624
	Gly Ser Thr Cys Ala Val Phe Gly Leu Gly Gly Val Gly Leu Ser Val	
	195 200 205	
5	ATC ATG GGC TGT AAA GCA GCC GGA GCG GCC AGG ATC ATT GGG GTG GAC	672
	Ile Met Gly Cys Lys Ala Ala Gly Ala Ala Arg Ile Ile Gly Val Asp	
	210 215 220	
10	ATC AAC AAA GAC AAG TTT GCA AAG GCC AAA GAA GTG GGT GCC ACT GAG	720
	Ile Asn Lys Asp Lys Phe Ala Lys Ala Lys Glu Val Gly Ala Thr Glu	
	225 230 235	
15	TGT GTC AAC CCT CAG GAC TAC AAG AAA CCC ATC CAG GAG GTG CTG ACA	768
	Cys Val Asn Pro Gln Asp Tyr Lys Lys Pro Ile Gln Glu Val Leu Thr	
	240 245 250 255	
20	GAA ATA AGC AAT GGA GGT GTG GAT TTT TCC TTT GAA GTC ATT GGT CGG	816
	Glu GCG Ser Asn Gly Gly Val Asp Phe Ser Phe Glu Val Ile Gly Arg	
	260 265 270	
25	CTC GAC ACT ATG GTG ACT GCC TTG TCA TGC TGT CAA GAA GCA TAT GGT	864
	Leu Asp Thr Met Val Thr Ala Leu Ser Cys Cys Gln Glu Ala Tyr Gly	
	275 280 285	
30	GTG AGC GTC ATT GCG GGA GTA CCT CCT GAT TCC CAA AAT CTC TCT ATG	912
	Val Ser Val Ile Ala Gly Val Pro Pro Asp Ser Gln Asn Leu Ser Met	
	290 295 300	
35	AAT CCT ATG TTG CTA CTG AGT GGA CGT ACC TGG AAA GGA GCT ATT TTT	960
	Asn Pro Met Leu Leu Leu Ser Gly Arg Thr Trp Lys Gly Ala Ile Phe	
	305 310 315	
40	GGC GGT TTT AAG AGT AAA GAT TCT GTC CCC AAA CTT GTG GCC GAT TTT	1008
	Gly Gly Phe Lys Ser Lys Asp Ser Val Pro Lys Leu Val Ala Asp Phe	
	320 325 330 335	
45	ATG GCT AAA AAG TTT GCA CTG GAT CCT TTA ATC ACC CAT GTT TTA CCT	1056
	Met Ala Lys Lys Phe Ala Leu Asp Pro Leu Ile Thr His Val Leu Pro	
	340 345 350	
50	TTT GAA AAA ATA AAT GAA GGA TTT GAC CTG CTT CGC TCT GGA GAG AGT	1104
	Phe Glu Lys Ile Asn Glu Gly Phe Asp Leu Leu Arg Ser Gly Glu Ser	
	355 360 365	
55	ATC CGT ACC ATC CTG ACG TTT TGA	1128
	Ile Arg Thr Ile Leu Thr Phe	
	370	
60	(2) INFORMATION FOR SEQ ID NO:14:	
	(i) SEQUENCE CHARACTERISTICS:	
	(A) LENGTH: 374 amino acids	
	(B) TYPE: amino acid	
	(D) TOPOLOGY: linear	
	(ii) MOLECULE TYPE: protein	
	(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:	
65	Ser Thr Ala Gly Lys Val Ile Lys Cys Lys Ala Ala Val Leu Trp Glu	
	1 5 10 15	
70	Glu Lys Lys Pro Phe Ser Ile Glu Glu Val Glu Val Ala Pro Pro Lys	
	20 25 30	
75	Ala His Glu Val Arg Ile Lys Met Val Ala Thr Gly Ile Cys Arg Ser	
	35 40 45	
80	Asp Asp His Val Val Ser Gly Thr Leu Val Thr Pro Leu Pro Val Ile	
	50 55 60	
85	Ala Gly His Glu Ala Ala Gly Ile Val Glu Ser Ile Gly Glu Gly Val	
	65 70 75 80	
90	Thr Thr Val Arg Pro Gly Asp Lys Val Ile Pro Leu Phe Thr Pro Gln	
	85 90 95	
95	Cys Gly Lys Cys Arg Val Cys Lys His Pro Glu Gly Asn Phe Cys Leu	
	100 105 110	
100	Lys Asn Asp Leu Ser Met Pro Arg Gly Thr Met Gln Asp Gly Thr Ser	
	115 120 125	

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Arg Phe Thr Cys Arg Gly Lys Pro Ile His His Phe Leu Gly Thr Ser  
 130 135 140  
 5 Thr Phe Ser Gln Tyr Thr Val Val Asp Glu Ile Ser Val Ala Lys Ile  
 145 150 155 160  
 Asp Ala Ala Ser Pro Leu Glu Lys Val Cys Leu Ile Gly Cys Gly Phe  
 165 170 175  
 10 Ser Thr Gly Tyr Gly Ser Ala Val Lys Val Ala Lys Val Thr Gln Gly  
 180 185 190  
 Ser Thr Cys Ala Val Phe Gly Leu Gly Gly Val Gly Leu Ser Val Ile  
 195 200 205  
 15 Met Gly Cys Lys Ala Ala Gly Ala Ala Arg Ile Ile Gly Val Asp Ile  
 210 215 220  
 Asn Lys Asp Lys Phe Ala Lys Ala Lys Glu Val Gly Ala Thr Glu Cys  
 225 230 235 240  
 20 Val Asn Pro Gln Asp Tyr Lys Lys Pro Ile Gln Glu Val Leu Thr Glu  
 245 250 255  
 25 Ile Ser Asn Gly Gly Val Asp Phe Ser Phe Glu Val Ile Gly Arg Leu  
 260 265 270  
 Asp Thr Met Val Thr Ala Leu Ser Cys Cys Gln Glu Ala Tyr Gly Val  
 275 280 285  
 30 Ser Val Ile Ala Gly Val Pro Pro Asp Ser Gln Asn Leu Ser Met Asn  
 290 295 300  
 35 Pro Met Leu Leu Leu Ser Gly Arg Thr Trp Lys Gly Ala Ile Phe Gly  
 305 310 315 320  
 Gly Phe Lys Ser Lys Asp Ser Val Pro Lys Leu Val Ala Asp Phe Met  
 325 330 335  
 40 Ala Lys Lys Phe Ala Leu Asp Pro Leu Ile Thr His Val Leu Pro Phe  
 340 345 350  
 Glu Lys Ile Asn Glu Gly Phe Asp Leu Leu Arg Ser Gly Glu Ser Ile  
 355 360 365  
 45 Arg Thr Ile Leu Thr Phe  
 370  
 50 (2) INFORMATION FOR SEQ ID NO:15:  
 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 1128 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: double  
 (D) TOPOLOGY: linear  
 55  
 (ii) MOLECULE TYPE: DNA (genomic)  
 60  
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:  
 ATG AGC ACA GCA GGA AAA GTA ATA AAA TGC AAA GCG GCT GTG CTG TGG 48  
 Ser Thr Ala Gly Lys Val Ile Lys Cys Lys Ala Ala Val Leu Trp 15  
 1 5 10  
 65 GAG GAA AAG AAA CCA TTT TCC ATC GAG GAG GTG GAG GTT GCA CCC CCG 96  
 Glu Glu Lys Lys Pro Phe Ser Ile Glu Glu Val Glu Val Ala Pro Pro 30  
 20 25 30  
 70 AAG GCC CAT GAA GTC CGT ATA AAG ATG GTG GCC ACA GGA ATT TGT CGC 144  
 Lys Ala His Glu Val Arg Ile Lys Met Val Ala Thr Gly Ile Cys Arg 35 40 45  
 75 TCA GAT GAC CAC GTG GTT AGT GGA ACC CTT GTC ACA CCT CTT CCT GTG 192  
 Ser Asp Asp His Val Val Ser Gly Thr Leu Val Thr Pro Leu Pro Val 50 55 60  
 80 ATC GCA GGC CAT GAG GCA GCG GGC ATT GTG GAG AGC ATT GGA GAA GGC 240  
 Ile Ala Gly His Glu Ala Gly Ile Val Glu Ser Ile Gly Glu Gly 65 70 75  
 GTC ACT ACA GTA AGA CCA GGT GAT AAA GTC ATC CCA CTC TTT ACT CCC 288  
 Val Thr Thr Val Arg Pro Gly Asp Lys Val Ile Pro Leu Phe Thr Pro

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	80	85	90	95	
5	CAG TGT GGA AAA TGC AGG GTT TGT AAG CAC CCT GAA GGC AAC TTC TGC Gln Cys Gly Lys Cys Arg Val Cys Lys His Pro Glu Gly Asn Phe Cys	100	105	110	336
10	TTG AAA AAT GAT CTG AGC ATG CCT CGG GGA ACC ATG CAG GAT GGT ACC Leu Lys Asn Asp Leu Ser Met Pro Arg Gly Thr Met Gln Asp Gly Thr	115	120	125	384
15	AGC AGG TTC ACC TGC AGA GGG AAG CCC ATC CAC CAC TTC CTT GGC ACC Ser Arg Phe Thr Cys Arg Gly Lys Pro Ile His His Phe Leu Gly Thr	130	135	140	432
20	AGC ACC TTC TCC CAG TAC ACC GTG GTG GAC GAG ATC TCA GTG GCC AAG Ser Thr Phe Ser Gln Tyr Thr Val Val Asp Glu Ile Ser Val Ala Lys	145	150	155	480
25	ATC GAT GCG GCC TCA CCG CTG GAG AAA GTC TGT CTC ATT GGC TGT GGA Ile Asp Ala Ala Ser Pro Leu Glu Lys Val Cys Leu Ile Gly Cys Gly	160	165	170	528
30	TTT ACT ACT GGT TAT GGG TCT GCA GTC AAG GTT GCC AAG GTC ACC CAG Phe Thr Thr Gly Tyr Gly Ser Ala Val Lys Val Ala Lys Val Thr Gln	180	185	190	576
35	GGC TCC ACC TGT GCC GTG TTT GGC CTT GGA GGA GTG GGC CTG TCT GTT Gly Ser Thr Cys Ala Val Phe Gly Leu Gly Gly Val Gly Leu Ser Val	195	200	205	624
40	ATC ATG GGC TGT AAA GCA GCC GGA GCG GCC AGG ATC ATT GCG GTG GAC Ile Met Gly Cys Lys Ala Ala Gly Ala Ala Arg Ile Ile Gly Val Asp	210	215	220	672
45	ATC AAC AAA GAC AAG TTT GCA AAG GCC AAA GAA GTG GST GCC ACT GAG Ile Asn Lys Asp Lys Phe Ala Lys Ala Lys Glu Val Gly Ala Thr Glu	225	230	235	720
50	TGT GTC AAC CCT CAG GAC TAC AAG AAA CCC ATC CAG GAG GTG CTG ACA Cys Val Asn Pro Gln Asp Tyr Lys Lys Pro Ile Gln Glu Val Leu Thr	240	245	250	768
55	GAA ATG AGC AAT GGA GGT GTG GAT TTT TCC TTT GAA GTC ATT GGT CGG Glu Met Ser Asn Gly Gly Val Asp Phe Ser Phe Glu Val Ile Gly Arg	260	265	270	816
60	CTC GAC ACT ATG GTG ACT GCC TTG TCA TGC TGT CAA GAA GCA TAT GGT Leu Asp Thr Met Val Thr Ala Leu Ser Cys Cys Gln Glu Ala Tyr Gly	275	280	285	864
65	GTG AGC GTC ATT GTG GGA GTA CCT CCT GAT TCC CAA AAT CTC TCT ATG Val Ser Val Ile Val Gly Val Pro Pro Asp Ser Gln Asn Leu Ser Met	290	295	300	912
70	AAT CCT ATG TTG CTA CTG AGT GGA CGT ACC TGG AAA GGA GCT ATT TTT Asn Pro Met Leu Leu Leu Ser Gly Arg Thr Trp Lys Gly Ala Ile Phe	305	310	315	960
75	GGC GGT TTT AAG AGT AAA GAT TCT GTC CCC AAA CTT GTG GCC GAT TTT Gly Gly Phe Lys Ser Lys Asp Ser Val Pro Lys Leu Val Ala Asp Phe	320	325	330	1008
80	ATG GCT AAA AAG TTT GCA CTG GAT CCT TTA ATC ACC CAT GTT TTA CCT Met Ala Lys Lys Phe Ala Leu Asp Pro Leu Ile Thr His Val Leu Pro	340	345	350	1056
	TTT GAA AAA ATA AAT GAA GGA TTT GAC CTG CTT CGC TCT GGA GAG AGT Phe Glu Lys Ile Asn Glu Gly Phe Asp Leu Leu Arg Ser Gly Glu Ser	355	360	365	1104
	ATC CGT ACC ATC CTG ACG TTT TGA Ile Arg Thr Ile Leu Thr Phe	370			1128

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 374 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

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## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

5 Ser Thr Ala Gly Lys Val Ile Lys Cys Lys Ala Ala Val Leu Trp Glu  
 1 5 10 15  
 Glu Lys Lys Pro Phe Ser Ile Glu Glu Val Glu Val Ala Pro Pro Lys  
 20 25 30  
 10 Ala His Glu Val Arg Ile Lys Met Val Ala Thr Gly Ile Cys Arg Ser  
 35 40 45  
 Asp Asp His Val Val Ser Gly Thr Leu Val Thr Pro Leu Pro Val Ile  
 50 55 60  
 15 Ala Gly His Glu Ala Ala Gly Ile Val Glu Ser Ile Gly Glu Gly Val  
 65 70 75 80  
 Thr Thr Val Arg Pro Gly Asp Lys Val Ile Pro Leu Phe Thr Pro Gln  
 85 90 95  
 20 Cys Gly Lys Cys Arg Val Cys Lys His Pro Glu Gly Asn Phe Cys Leu  
 100 105 110  
 Lys Asn Asp Leu Ser Met Pro Arg Gly Thr Met Gln Asp Gly Thr Ser  
 115 120 125  
 25 Arg Phe Thr Cys Arg Gly Lys Pro Ile His His Phe Leu Gly Thr Ser  
 130 135 140  
 30 Thr Phe Ser Gln Tyr Thr Val Val Asp Glu Ile Ser Val Ala Lys Ile  
 145 150 155 160  
 Asp Ala Ala Ser Pro Leu Glu Lys Val Cys Leu Ile Gly Cys Gly Phe  
 165 170 175  
 35 Thr Thr Gly Tyr Gly Ser Ala Val Lys Val Ala Lys Val Thr Gln Gly  
 180 185 190  
 40 Ser Thr Cys Ala Val Phe Gly Leu Gly Gly Val Gly Leu Ser Val Ile  
 195 200 205  
 Met Gly Cys Lys Ala Ala Gly Ala Ala Arg Ile Ile Gly Val Asp Ile  
 210 215 220  
 45 Asn Lys Asp Lys Phe Ala Lys Ala Lys Glu Val Gly Ala Thr Glu Cys  
 225 230 235 240  
 Val Asn Pro Gln Asp Tyr Lys Lys Pro Ile Gln Glu Val Leu Thr Glu  
 245 250 255  
 50 Met Ser Asn Gly Gly Val Asp Phe Ser Phe Glu Val Ile Gly Arg Leu  
 260 265 270  
 55 Asp Thr Met Val Thr Ala Leu Ser Cys Cys Gln Glu Ala Tyr Gly Val  
 275 280 285  
 Ser Val Ile Val Gly Val Pro Pro Asp Ser Gln Asn Leu Ser Met Asn  
 290 295 300  
 60 Pro Met Leu Leu Leu Ser Gly Arg Thr Trp Lys Gly Ala Ile Phe Gly  
 305 310 315 320  
 Gly Phe Lys Ser Lys Asp Ser Val Pro Lys Leu Val Ala Asp Phe Met  
 325 330 335  
 65 Ala Lys Lys Phe Ala Leu Asp Pro Leu Ile Thr His Val Leu Pro Phe  
 340 345 350  
 70 Glu Lys Ile Asn Glu Gly Phe Asp Leu Leu Arg Ser Gly Glu Ser Ile  
 355 360 365  
 Arg Thr Ile Leu Thr Phe  
 370

## (2) INFORMATION FOR SEQ ID NO:17:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1128 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: double  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

5	ATG AGC ACA GCA GGA AAA GTA ATA AAA TGC AAA GCG GCT GTG CTG TGG Ser Thr Ala Gly Lys Val Ile Lys Cys Lys Ala Ala Val Leu Trp 1 5 10 15	48
10	GAG GAA AAG AAA CCA TTT TCC ATC GAG GAG GTG GAG GTT GCA CCC CCG Glu Glu Lys Lys Pro Phe Ser Ile Glu Glu Val Glu Val Ala Pro Pro 20 25 30	96
15	AAG GCC CAT GAA GTC CGT ATA AAG ATG GTG GCT ACA GGA ATT TGT CGC Lys Ala His Glu Val Arg Ile Lys Met Val Ala Thr Gly Ile Cys Arg 35 40 45	144
20	TCA GAT GAC CAC GTA GTT AGT GGA ACC CTT GTC ACA CCT CTT CCT GTG Ser Asp Asp His Val Val Ser Gly Thr Leu Val Thr Pro Leu Pro Val 50 55 60	192
25	ATC GCA GGC CAT GAG GCA GCG GGC ATT GTG GAG AGC ATT GGA GAA GGC Ile Ala Gly His Glu Ala Ala Gly Ile Val Glu Ser Ile Gly Glu Gly 65 70 75	240
30	GTC ACT ACA GTA AGA CCA GGT GAT AAA GTC ATC CCA CTC TTT ACT CCC Val Thr Thr Val Arg Pro Gly Asp Lys Val Ile Pro Leu Phe Thr Pro 80 85 90 95	288
35	CAG TGT GGA AAA TGC AGG GTT TGT AAG CAC CCT GAA GGC AAC CTC TGC Gln Cys Gly Lys Cys Arg Val Cys Lys His Pro Glu Gly Asn Leu Cys 100 105 110	336
40	TTG AAA AAT GAT CTG AGC ATG CCT CGG GGA ACC ATG CAG GAT GGT ACC Leu Lys Asn Asp Leu Ser Met Pro Arg Gly Thr Met Gln Asp Gly Thr 115 120 125	384
45	AGC AGG TTC ACC TGC AGA GGG AAG CCC ATC CAC CAC TTC CTT GGC ACC Ser Arg Phe Thr Cys Arg Gly Lys Pro Ile His His Phe Leu Gly Thr 130 135 140	432
50	AGC ACC TTC TCC CAG TAC ACC GTG GTG GAC GAG ATC TCA GTG GCC AAG Ser Thr Phe Ser Gln Tyr Thr Val Val Asp Glu Ile Ser Val Ala Lys 145 150 155	480
55	ATC GAT GCG GCC TCA CCG CTG GAG AAA GTC TGT CTC ATT GGC TGT GGA Ile Asp Ala Ala Ser Pro Leu Glu Lys Val Cys Leu Ile Gly Cys Gly 160 165 170 175	528
60	TTT TCT ACT GGT TAT GGG TCT GCA GTC AAG GTT GCC AAG GTC ACC CAG Phe Ser Thr Gly Tyr Gly Ser Ala Val Lys Val Ala Lys Val Thr Gln 180 185 190	576
65	GGC TCC ACC TGT GCC GTG TTT GGC CTT GGA GGA GTG GGC CTG TCT GTT Gly Ser Thr Cys Ala Val Phe Gly Leu Gly Gly Val Gly Leu Ser Val 195 200 205	624
70	ATC ATG GGC TGT AAA GCA GCC GGA GCG GCC AGG ATC ATT GGG GTG GAC Ile Met Gly Cys Lys Ala Ala Gly Ala Ala Arg Ile Ile Gly Val Asp 210 215 220	672
75	ATC AAC AAA GAC AAG TTT GCA AAG GCC AAA GAA GTG GGT GCC ACT GAG Ile Asn Lys Asp Lys Phe Ala Lys Ala Lys Glu Val Gly Ala Thr Glu 225 230 235	720
80	TGT GTC AAC CCT CAG GAC TAC AAG AAA CCC ATC CAG GAG GTG CTG ACA Cys Val Asn Pro Gln Asp Tyr Lys Lys Pro Ile Gln Glu Val Leu Thr 240 245 250 255	768
85	GAA ATG AGC AAT GGA GGT GTG GAT TTT TCC TTT GAA GTC ATT GGT CGG Glu Met Ser Asn Gly Gly Val Asp Phe Ser Phe Glu Val Ile Gly Arg 260 265 270	816
90	CTC GAC ACT ATG GTG ACT GCC TTG TCA TGC TGT CAA GAA GCA TAT GGT Leu Asp Thr Met Val Thr Ala Leu Ser Cys Cys Gln Glu Ala Tyr Gly 275 280 285	864
95	GTG AGC GTC ATT GTG GGA GTA CCT CCT GAT TCC CAA AAT CTC TCT ATG Val Ser Val Ile Val Gly Val Pro Pro Asp Ser Gln Asn Leu Ser Met 290 295 300	912
100	AAT CCT ATG TTG CTA CTG AGT GGA CGT ACC TGG AAA GGA GCT ATT TTT Asn Pro Met Leu Leu Leu Ser Gly Arg Thr Trp Lys Gly Ala Ile Phe 305 310 315	960

53

GGC GGT TTT AAG AGT AAA GAT TCT GTC CCC AAA CTT GTG GCC GAT TTT 1008  
 Gly Gly Phe Lys Ser Lys Asp Ser Val Pro Lys Leu Val Ala Asp Phe  
 320 325 330 335  
 5 ATG GCT AAA AAG TTT GCA CTG GAT CCT TTA ATC ACC CAT GTT TTA CCT 1056  
 Met Ala Lys Lys Phe Ala Leu Asp Pro Leu Ile Thr His Val Leu Pro  
 340 345 350  
 10 TTT GAA AAA ATA AAT GAA GGA TTT GAC CTG CTT CGC TCT GGA GAG AGT 1104  
 Phe Glu Lys Ile Asn Glu Gly Phe Asp Leu Leu Arg Ser Gly Glu Ser  
 355 360 365  
 ATC CGT ACC ATC CTG ACG TTT TGA 1128  
 Ile Arg Thr Ile Leu Thr Phe  
 370

(2) INFORMATION FOR SEQ ID NO:18:

20

(i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 374 amino acids  
 (B) TYPE: amino acid  
 (D) TOPOLOGY: linear

25

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

30 Ser Thr Ala Gly Lys Val Ile Lys Cys Lys Ala Ala Val Leu Trp Glu  
 1 5 10 15  
 Glu Lys Lys Pro Phe Ser Ile Glu Glu Val Glu Val Ala Pro Pro Lys  
 20 25 30  
 35 Ala His Glu Val Arg Ile Lys Met Val Ala Thr Gly Ile Cys Arg Ser  
 35 40 45  
 Asp Asp His Val Val Ser Gly Thr Leu Val Thr Pro Leu Pro Val Ile  
 50 55 60  
 40 Ala Gly His Glu Ala Ala Gly Ile Val Glu Ser Ile Gly Glu Gly Val  
 65 70 75 80  
 Thr Thr Val Arg Pro Gly Asp Lys Val Ile Pro Leu Phe Thr Pro Gln  
 85 90 95  
 45 Cys Gly Lys Cys Arg Val Cys Lys His Pro Glu Gly Asn Leu Cys Leu  
 100 105 110  
 50 Lys Asn Asp Leu Ser Met Pro Arg Gly Thr Met Gln Asp Gly Thr Ser  
 115 120 125  
 Arg Phe Thr Cys Arg Gly Lys Pro Ile His His Phe Leu Gly Thr Ser  
 130 135 140  
 55 Thr Phe Ser Gln Tyr Thr Val Val Asp Glu Ile Ser Val Ala Lys Ile  
 145 150 155 160  
 60 Asp Ala Ala Ser Pro Leu Glu Lys Val Cys Leu Ile Gly Cys Gly Phe  
 165 170 175  
 Ser Thr Gly Tyr Gly Ser Ala Val Lys Val Ala Lys Val Thr Gln Gly  
 180 185 190  
 65 Ser Thr Cys Ala Val Phe Gly Leu Gly Gly Val Gly Leu Ser Val Ile  
 195 200 205  
 Met Gly Cys Lys Ala Ala Gly Ala Ala Arg Ile Ile Gly Val Asp Ile  
 210 215 220  
 70 Asn Lys Asp Lys Phe Ala Lys Ala Lys Glu Val Gly Ala Thr Glu Cys  
 225 230 235 240  
 Val Asn Pro Gln Asp Tyr Lys Lys Pro Ile Gln Glu Val Leu Thr Glu  
 245 250 255  
 75 Met Ser Asn Gly Gly Val Asp Phe Ser Phe Glu Val Ile Gly Arg Leu  
 260 265 270  
 80 Asp Thr Met Val Thr Ala Leu Ser Cys Cys Gln Glu Ala Tyr Gly Val  
 275 280 285  
 Ser Val Ile Val Gly Val Pro Pro Asp Ser Gln Asn Leu Ser Met Asn  
 290 295 300

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	Pro Met Leu Leu Leu Ser Gly Arg Thr Trp Lys Gly Ala Ile Phe Gly	
	305 310 315 320	
5	Gly Phe Lys Ser Lys Asp Ser Val Pro Lys Leu Val Ala Asp Phe Met	
	325 330 335	
	Ala Lys Lys Phe Ala Leu Asp Pro Leu Ile Thr His Val Leu Pro Phe	
10	340 345 350	
	Glu Lys Ile Asn Glu Gly Phe Asp Leu Leu Arg Ser Gly Glu Ser Ile	
	355 360 365	
15	Arg Thr Ile Leu Thr Phe	
	370	
	(2) INFORMATION FOR SEQ ID NO:19:	
20	(i) SEQUENCE CHARACTERISTICS:	
	(A) LENGTH: 1128 base pairs	
	(B) TYPE: nucleic acid	
	(C) STRANDEDNESS: double	
25	(D) TOPOLOGY: linear	
	(ii) MOLECULE TYPE: DNA (genomic)	
30	(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:	
	ATG AGC ACA GCA GGA AAA GTA ATA AAA TGC AAA GCG GCT GTG CTG TGG	48
	Ser Thr Ala Gly Lys Val Ile Lys Cys Lys Ala Ala Val Leu Trp	
	1 5 10 15	
35	GAG GAA AAG AAA CCA TTT TCC ATC GAG GAG GTG GAG GTT GCA CCC CCG	96
	Glu Glu Lys Lys Pro Phe Ser Ile Glu Glu Val Glu Val Ala Pro Pro	
	20 25 30	
40	AAG GCC CAT GAA GTC CGT ATA AAG ATG GTG NNN ACA GGA ATT TGT CGC	144
	Lys Ala His Glu Val Arg Ile Lys Met Val Ala Thr Gly Ile Cys Arg	
	35 40 45	
45	TCA GAT GAC CAC NNN GTT AGT GGA ACC CTT GTC ACA CCT CTT CCT GTG	192
	Ser Asp Asp His Val Val Ser Gly Thr Leu Val Thr Pro Leu Pro Val	
	50 55 60	
50	ATC GCA GGC CAT GAG GCA GCG GGC ATT GTG GAG NNN ATT GGA GAA GGC	240
	Ile Ala Gly His Glu Ala Ala Gly Ile Val Glu Xaa Ile Gly Glu Gly	
	65 70 75	
55	GTC ACT ACA GTA AGA CCA GGT GAT AAA GTC ATC CCA CTC TTT NNN CCC	288
	Val Thr Thr Val Arg Pro Gly Asp Lys Val Ile Pro Leu Phe Xaa Pro	
	80 85 90 95	
60	CAG TGT GGA AAA TCC AGG GTT TGT AAG CAC CCT GAA GGC AAC NNN TGC	336
	Gln Cys Gly Lys Cys Arg Val Cys Lys His Pro Glu Gly Asn Xaa Cys	
	100 105 110	
65	TTG AAA AAT GAT CTG AGC ATG CCT CGG GGA ACC ATG CAG GAT GGT ACC	384
	Leu Lys Asn Asp Leu Ser Met Pro Arg Gly Thr Met Gln Asp Gly Thr	
	115 120 125	
70	AGC AGG TTC ACC TGC AGA GGG AAG CCC ATC CAC CAC TTC CTT GGC ACC	432
	Ser Arg Phe Thr Cys Arg Gly Lys Pro Ile His His Phe Leu Gly Thr	
	130 135 140	
75	AGC ACC TTC TCC CAG TAC ACC GTG GTG GAC GAG ATC TCA GTG GCC AAG	480
	Ser Thr Phe Ser Gln Tyr Thr Val Val Asp Glu Ile Ser Val Ala Lys	
	145 150 155	
80	ATC GAT GCG GCC TCA CCG CTG GAG AAA GTC TGT CTC ATT GGC TGT GGA	528
	Ile Asp Ala Ala Ser Pro Leu Glu Lys Val Cys Leu Ile Gly Cys Gly	
	160 165 170 175	
85	TTT NNN ACT GGT TAT GGG TCT GCA GTC AAG GTT GCC AAG GTC ACC CAG	576
	Phe Xaa Thr Gly Tyr Gly Ser Ala Val Lys Val Ala Lys Val Thr Gln	
	180 185 190	
90	GGC TCC ACC TGT GCC GTG TTT GGC CTT GGA GGA GTG GGC CTG TCT GTT	624
	Gly Ser Thr Cys Ala Val Phe Gly Leu Gly Gly Val Gly Leu Ser Val	
	195 200 205	
95	ATC ATG GGC TGT AAA GCA GCC GGA GCG GCC AGG ATC ATT GGG GTG GAC	672
	Ile Met Gly Cys Lys Ala Ala Gly Ala Ala Arg Ile Ile Gly Val Asp	



55

	210	215	220	
5	ATC AAC AAA GAC AAG TTT Ile Asn Lys Asp Lys Phe 225	GCA AAG GCC AAA GAA GTG Ala Lys Ala Lys Glu Val 230	GGT GCC ACT GAG Gly Ala Thr Glu 235	720
10	TGT GTC AAC CCT CAG GAC Cys Val Asn Pro Gln Asp Tyr 240	TAC AAG AAA CCC ATC Lys Lys Lys Pro Ile 245	CAG GAG GTG CTG ACA Gln Glu Val Leu Thr 250	768
15	GAA NNN AGC AAT GGA GGT GTG Glu Xaa Ser Asn Gly Gly Val 260	GAT TTT TCC TTT GAA NNN Asp Phe Ser Phe Glu Xaa 265	ATT GGT CGG Ile Gly Arg 270	816
20	CTC GAC ACT ATG GTG ACT Leu Asp Thr Met Val Thr 275	GCC TTG TCA TGC NNN Ala Leu Ser Cys Xaa 280	GAA GCA TAT GGT Gln Glu Ala Tyr Gly 285	864
25	GTG AGC GTC ATT NNN GGA Val Ser Val Ile Xaa Gly Val 290	GTA CCT CCT NNN TCC Pro Pro Xaa Ser Gln 295	CAA AAT CTC TCT ATG Asn Leu Ser Met 300	912
30	AAT CCT ATG TTG CTA CTG Asn Pro Met Leu Leu Leu 305	AGT GGA CGT ACC TGG Ser Gly Arg Thr Trp 310	AAA GGA GCT ATT TTT Lys Gly Ala Ile Phe 315	960
35	GGC GGT TTT AAG AGT AAA Gly Gly Phe Lys Ser Lys 320	GAT TCT GTC CCC AAA Asp Ser Val Pro Lys 325	CTT GTG GCC GAT TTT Leu Val Ala Asp Phe 330	1008
40	ATG GCT AAA AAG TTT GCA Met Ala Lys Lys Phe Ala 340	CTG GAT CCT TTA ATC Leu Asp Pro Leu Ile Thr 345	ACC CAT GTT TTA CCT His Val Leu Pro 350	1056
45	TTT GAA AAA ATA AAT GAA Phe Glu Lys Ile Asn Glu 355	GGA TTT GAC CTG CTT Gly Phe Asp Leu Leu 360	CGC TCT GGA GAG AGT Ser Gly Glu Ser 365	1104
50	ATC CGT ACC ATC CTG ACG Ile Arg Thr Ile Leu Thr 370	TTT TGA Phe 375		1128

## (2) INFORMATION FOR SEQ ID NO:20:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 374 amino acids
  - (B) TYPE: amino acid
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

55	Ser Thr Ala Gly Lys Val Ile Lys Cys Lys Ala Ala Val Leu Trp Glu 1 5 10 15
60	Glu Lys Lys Pro Phe Ser Ile Glu Glu Val Glu Val Ala Pro Pro Lys 20 25 30
65	Ala His Glu Val Arg Ile Lys Met Val Xaa Thr Gly Ile Cys Arg Ser 35 40 45
70	Asp Asp His Xaa Val Ser Gly Thr Leu Val Thr Pro Leu Pro Val Ile 50 55 60
75	Ala Gly His Glu Ala Ala Gly Ile Val Glu Xaa Ile Gly Glu Gly Val 65 70 75 80
80	Thr Thr Val Arg Pro Gly Asp Lys Val Ile Pro Leu Phe Xaa Pro Gln 85 90 95
	Cys Gly Lys Cys Arg Val Cys Lys His Pro Glu Gly Asn Xaa Cys Leu 100 105 110
	Lys Asn Asp Leu Ser Met Pro Arg Gly Thr Met Gln Asp Gly Thr Ser 115 120 125
	Arg Phe Thr Cys Arg Gly Lys Pro Ile His His Phe Leu Gly Thr Ser 130 135 140
	Thr Phe Ser Gln Tyr Thr Val Val Asp Glu Ile Ser Val Ala Lys Ile 145 150 155 160

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Asp Ala Ala Ser Pro Leu Glu Lys Val Cys Leu Ile Gly Cys Gly Phe  
 165 170 175  
 5 Xaa Thr Gly Tyr Gly Ser Ala Val Lys Val Ala Lys Val Thr Gln Gly  
 180 185 190  
 Ser Thr Cys Ala Val Phe Gly Leu Gly Gly Val Gly Leu Ser Val Ile  
 195 200 205  
 10 Met Gly Cys Lys Ala Ala Gly Ala Ala Arg Ile Ile Gly Val Asp Ile  
 210 215 220  
 Asn Lys Asp Lys Phe Ala Lys Ala Lys Glu Val Gly Ala Thr Glu Cys  
 225 230 235 240  
 15 Val Asn Pro Gln Asp Tyr Lys Lys Pro Ile Gln Glu Val Leu Thr Glu  
 245 250 255  
 Xaa Ser Asn Gly Gly Val Asp Phe Ser Phe Glu Xaa Ile Gly Arg Leu  
 260 265 270  
 20 Asp Thr Met Val Thr Ala Leu Ser Cys Xaa Gln Glu Ala Tyr Gly Val  
 275 280 285  
 25 Ser Val Ile Xaa Gly Val Pro Pro Xaa Ser Gln Asn Leu Ser Met Asn  
 290 295 300  
 Pro Met Leu Leu Leu Ser Gly Arg Thr Trp Lys Gly Ala Ile Phe Gly  
 305 310 315 320  
 30 Gly Phe Lys Ser Lys Asp Ser Val Pro Lys Leu Val Ala Asp Phe Met  
 325 330 335  
 35 Ala Lys Lys Phe Ala Leu Asp Pro Leu Ile Thr His Val Leu Pro Phe  
 340 345 350  
 Glu Lys Ile Asn Glu Gly Phe Asp Leu Leu Arg Ser Gly Glu Ser Ile  
 355 360 365  
 40 Arg Thr Ile Leu Thr Phe  
 370

(2) INFORMATION FOR SEQ ID NO:21:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 31 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

CCCCGAATTC TCAAAACGTC AGGATGGTAC G

31

(2) INFORMATION FOR SEQ ID NO:22:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 44 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

CCCCTCTAGA ATAAATGAGC ACAGCAGGAA AAGTAATAAA ATGC

44

WHAT IS CLAIMED IS:

1. A method of obtaining a nonnative protein having a thermostability that is increased over that of the native version of said protein, wherein said method  
5 comprises:
  - (a) obtaining in a vector a gene that encodes said native protein;
  - (b) mutating said vector at more than one position in said gene to produce a vector library of cells  
10 comprising mutated versions of said gene;
  - (c) introducing said vector library *en masse* into cells of a strain in which the majority of said mutated versions of said gene are transcribed and translated to produce a cell library;
  - 15 (d) screening said cell library to identify a cell comprising a mutated version of said gene that encodes a nonnative protein having a thermostability that is increased over that of the wild-type version of said protein; and
  - 20 (e) purifying said cell from said cell library.
2. The method of claim 1 which further comprises isolating from said cell in a vector said mutated version of said gene and, on said mutated version of said gene,  
25 repeating steps (b) through (e).
3. The method of claim 1 wherein said protein is an alcohol dehydrogenase.
4. The method of claim 1 wherein said protein is horse liver alcohol dehydrogenase.
- 30 5. The method of claim 1, wherein said screen is carried out in the presence of alcohol.
6. The method of claim 1, wherein said screen is carried out at an increased temperature.
7. The method of claim 1, wherein said strain is  
35 either *Escherichi coli* or *Thermus flavus*.
8. A method for selecting against growth of *Escherichi coli* recombinant cells which comprise levels

of alcohol dehydrogenase that are higher than those of wild-type *Escherichia coli* cells, wherein said method comprises growing said recombinant cells under conditions selected from the group consisting of wherein ethanol is present in a concentration of about 10%, isopropanol is present in a concentration of about 4%, and propanol is present in a concentration of about 2%, with the proviso that said wild-type cells exhibit reduced or an absence of growth under said conditions.

9. A method for selecting for growth of *Thermus flavus* recombinant cells which comprise levels of alcohol dehydrogenase that are higher than those of wild-type *Thermus flavus* cells, wherein said method comprises growing said recombinant cells under conditions selected from the group consisting of wherein ethanol is present in a concentration of about 1% in a liquid or solid medium at a pH of about 7.0, and isopropanol is present in a concentration of from about 0.5% to about 1% in a liquid or solid medium at a pH of about 7.0, with the proviso that said wild-type cells exhibit reduced or an absence of growth under said conditions.

10. A method of increasing the thermostability of horse liver alcohol dehydrogenase, which comprises introducing into a gene which encodes said alcohol dehydrogenase a mutation at a codon which codes for an amino acid residue at a position selected from the group consisting of amino acid positions 75, 94, 110, 177, 257, 268, 282, 292, and 297.

11. A method of increasing the thermostability of horse liver alcohol dehydrogenase, which comprises changing an amino acid residue at a position selected from the group consisting of amino acid positions 75, 94, 110, 177, 257, 268, 282, 292, and 297.

12. An isolated and purified nucleic acid comprising a sequence selected from the group consisting of SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9,

SEQ ID NO:11, SEQ ID NO:13, SEQ ID NO:15, SEQ ID NO:17,  
and SEQ ID NO:19.

13. An isolated and purified protein comprising a  
sequence selected from the group consisting of SEQ ID  
5 NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, SEQ ID  
NO:12, SEQ ID NO:14, SEQ ID NO:16, SEQ ID NO:18, and SEQ  
ID NO:20.

14. A plasmid comprising the nucleic acid sequence  
of claim 12.

10 15. A plasmid selected from the group consisting of  
pAD7, pAD8, pAD10, pAD91, pAD92, pAD93, pAD95, pAD111,  
pAD113, and pTG450.

15 16. A vector library comprising an isolated and  
purified mixture of vectors comprising mutated versions  
of a horse liver alcohol dehydrogenase gene.

17. A host cell comprising a plasmid according to  
claim 14.

18. A host cell comprising a plasmid according to  
claim 15.

20 19. A host cell according to claim 17, wherein said  
cell is a member of the genus of *Thermus* or *Escherichia*.

20. A host cell according to claim 18, wherein said  
cell is strain TGF650.

25 21. A cell library comprising an isolated and  
purified mixture of cells obtained by transformation en  
masse with the vector library of claim 16.

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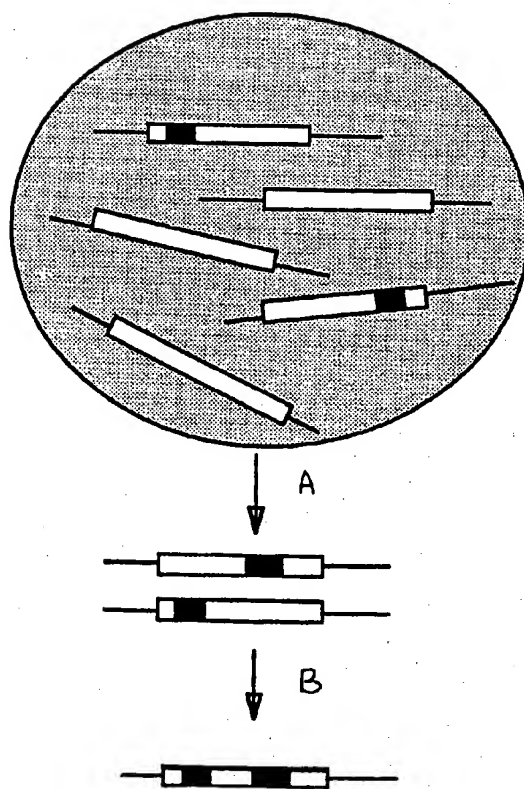


Fig 1

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Fig. 2A

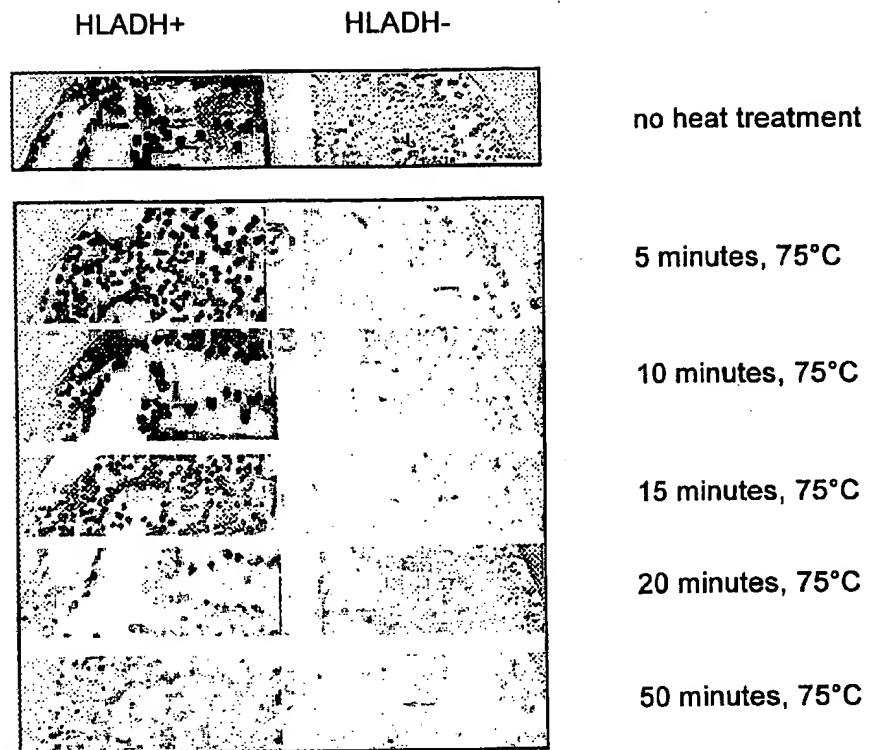


Fig. 2B

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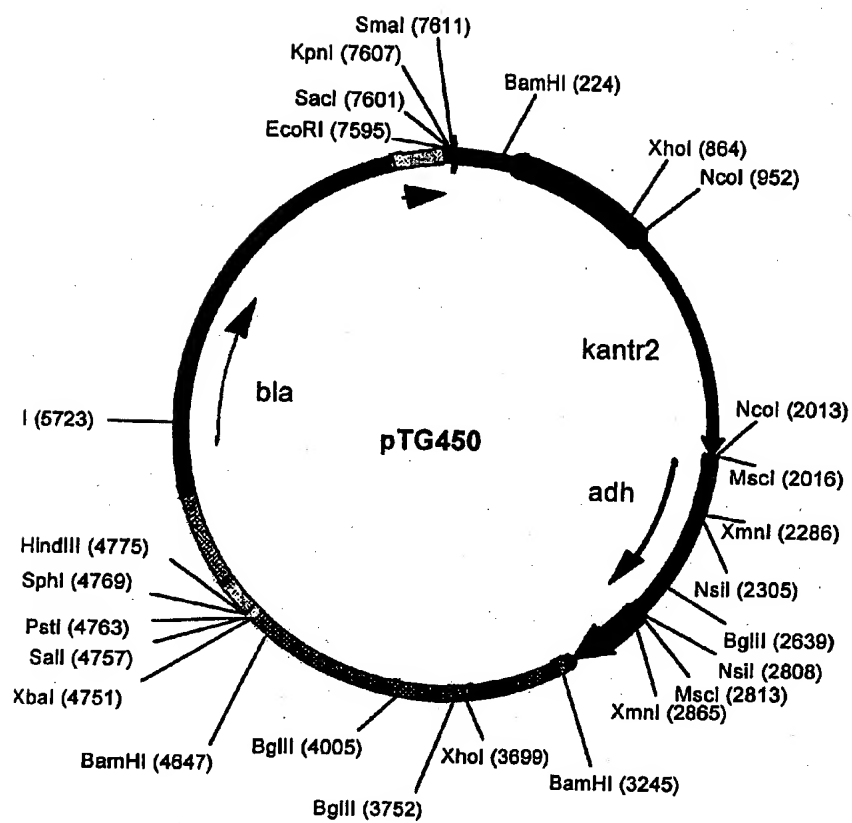


Fig. 3



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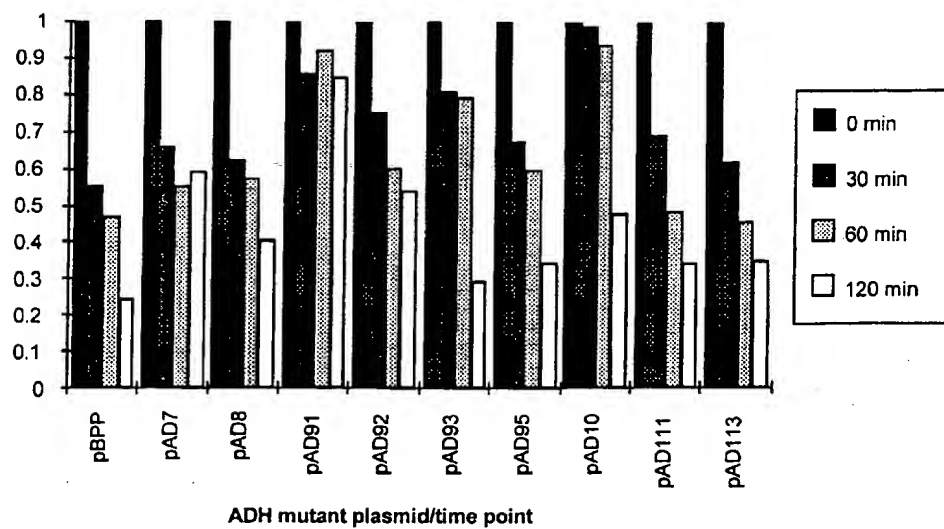


Fig. 4

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1 ATG AGC ACA GCA GGA AAA GTA ATA AAA TCC AAA GCG GCT GTG CTG TCG GAG GAA AAG AAA  
 1 Ser Thr Ala Gly Lys Val Ile Lys Cys Lys Ala Ala Val Val Leu Trp Glu GAG GAA AAG AAA  
 61 CCA TTT TCC ATC GAG GAG GTG GAG GTT GAG GAG GAG GAG GAG GAG GAG GAG GAG GAG GAG  
 20 Pro Phe Ser Ile Glu Val Val Glu Val Val Glu Val Val Glu Val Val Glu Val Val Glu Val  
 121 ATG GTG GCG ACA GGA ATT TGT CGC TCA GAT GAC CAC CAC CAC CAC CAC CAC CAC CAC CAC  
 40 Met Val Ala Thr Gly Ile Cys Arg Ser Asp Asp Asp Asp Asp Asp Asp Asp Asp Asp Asp  
 181 CCT CTT CTT CTT CTT CTT CTT CTT CTT CTT CTT CTT CTT CTT CTT CTT CTT CTT CTT CTT  
 60 Pro Leu Pro Val Ile Ala Gly His Glu Ala Ala Ala Ala Ala Ala Ala Ala Ala Ala Ala  
 241 GTC ACT ACA GTA AGA CCA GGT GAT AAA GTC Val Ile TGC TTG AAA AAT GAT CTG AGC ATG CCT  
 80 Val Thr Thr Val Arg Pro Gly Asp Lys Val Ile TGC TTG AAA AAT GAT CTG AGC ATG CCT  
 301 TGC AGG GTT TGT TGT TGT TGT TGT TGT TGT TGT TGT TGT TGT TGT TGT TGT TGT TGT TGT  
 100 Cys Arg Val Cys Lys His Pro Glu Gly Asn Phe Cys Leu Lys Asn Asp Leu Ser Met Pro  
 361 CGG GGA ACC ATG CAG GAT GGT ACC ACC AGC AGG TTC ACC TGC AGA GGG AAG CCC ATC CAC CAC  
 120 Arg Gly Thr Met Gln Asp Gly Thr Ser Arg Phe Thr Cys Arg Gly Lys Pro Ile His His  
 421 TTC CTT GGC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC  
 140 Phe Leu Gly Thr Ser Thr Phe Ser Thr Cys Arg Gly Lys Pro Ile His His  
 481 ATC GAT GCG GCC TCA CCG CTG GAG AAA GTC TGT CTC ATT GGC TGT GGA TTT TCT ACT GGT  
 160 Ile Asp Ala Ala Ser Pro Leu Gly Lys Val Cys Leu Ile Gly Cys Gly Phe Ser Thr Gly  
 541 TAT GGG TCT GCA GTC AAG GTT GCC AAG GTC ACC CAG GCG TCC ACC TGT GCC TGT GGT TTT  
 180 Tyr Gly Ser Ala Val Lys Val Ala Lys Val Thr Gln Gly Ser Thr Cys Ala Val Phe Thr  
 601 CTT GGA GGA GTG GCG CTG TCT GTT ATC ATG GCG TGT AAA GCA GCG GCG GCG GCG GCG GCG  
 200 Leu Gly Gly Val Gly Leu Ser Val Ile Met Gly Cys Lys Ala Ala Gly Ala Ala Arg Ile  
 661 ATT GGG GTG GAC ATC AAC AAA GAC AAG TTT GCA AAG GCC AAA GAA GTG GGT GCC ACT GAG  
 220 Ile Gly Val Asp Ile Asn Lys Asp Lys Phe Ala Lys Ala Lys Glu Val Gly Ala Thr Glu  
 721 TGT GTC AAC CCT CAG GAC TAC AAG AAA CCC ATC CAG GAG GTG CTG ACA GAA ATG AGC AAT  
 240 Cys Val Asn Pro Gln Asp Tyr Lys Lys Lys Lys Lys Lys Lys Lys Lys Lys Lys Lys Lys  
 781 GGA GGT GTG GAT TTT TCC TTT GAA GTC ATT GGT CCG CTC GAC ACT ATG GTG ACT GCG TTT  
 260 Gly Gly Val Asp Phe Ser Phe Glu Val Ile Gly Arg Leu Asp Thr Met Val Thr Ala Leu  
 841 TCA TCC TGT CAA GAA GCA TAT GGT GTG AGC GTC ATT GTG GGA GTA CCT CCT GAT TCC CAA  
 280 Ser Cys Cys Glu Ala Tyr Gly Val Ser Val Ile Val Val Val Val Val Val Val Val Val  
 901 AAT CTC TCT ATG AAT CCT ATG TTG CTA CTG AGT GGA CCG ACC TCG AAA GGA GCT ATT TTT  
 300 Asn Leu Ser Met Asn Pro Met Leu Leu Leu Ser Gly Arg Thr Trp Lys Gly Ala Ile Phe  
 961 GGC GGT TTT AAG AGT AAA GAT TCT TCT GTC CCC AAA CTT GTG GCG GAT TTT ATG GCT AAA AAG  
 320 Gly Gly Phe Lys Ser Lys Asp Ser Val Pro Lys Leu Val Ala Asp Phe Met Ala Lys Lys  
 1021 TTT GCA CTG GAT CCT TTA ATC ACC CAT GTT TTA CCT TTT GAA AAA ATA AAT GAA GGA TTT  
 340 Phe Ala Leu Asp Pro Leu Ile Thr His Val Leu Pro Phe Glu Lys Ile Asn Glu Gly Phe  
 1081 GAC CTG CTT CGC TCT GGA GAG AGT ATC CGT ACC ATC CTG ACG TTT TGA LSEG 1A No: 1J  
 360 Asp Leu Leu Arg Ser Gly Glu Ser Ile Arg Thr Ile Leu Thr Phe ... [Seq 2A No: 2J]

Fig. 5

## INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 98/09627

A. CLASSIFICATION OF SUBJECT MATTER  
IPC 6 C12N15/53 C12N9/04 C12N1/21

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	RELLOS P ET AL: "Polymerase chain reaction-based random mutagenesis: production and characterization of thermostable mutants of Zymomonas mobilis alcohol dehydrogenase-2." PROTEIN EXPR PURIF, JUN 1994, 5 (3) P270-7, XP002076286 UNITED STATES	1-3,5-8
Y	see page 271; figure 2 ---	4
Y	DE BOLLE X ET AL: "Identification of residues potentially involved in the interactions between subunits in yeast alcohol dehydrogenases." EUR J BIOCHEM, JUL 1 1995, 231 (1) P214-9, XP002076287 GERMANY see the whole document ---	4

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Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

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Date of the actual completion of the international search

2 September 1998

Date of mailing of the international search report

21/09/1998

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Authorized officer

Espen, J

# INTERNATIONAL SEARCH REPORT

International Application No.

PCT/US 98/09627

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>PARK DH ET AL: "Interconversion of E and S isoenzymes of horse liver alcohol dehydrogenase. Several residues contribute indirectly to catalysis."  J BIOL CHEM, MAR 15 1992, 267 (8)  P5527-33, XP002076288  UNITED STATES  see table 1</p>	<p>10-14,  16-19,21</p>
X	<p>PARK DH ET AL: "isoenzymes of horse liver alcohol dehydrogenase active on ethanol and steroids"  JOURNAL OF BIOLOGICAL CHEMISTRY,  vol. 266, 1991, pages 13296-13302,  XP002076289  MD US  see figure 3; tables I,VI</p>	<p>12,13</p>
A	<p>WEBER JM ET AL: "A chromosome integration system for stable gene transfer into Thermus flavus."  BIOTECHNOLOGY (N Y), MAR 1995, 13 (3)  P271-5, XP002076290  UNITED STATES</p>	